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13. ABSTRACT (Maximum 200 Words)

We found that expression of the pro-angiogenic gene vascular endothelial growth factor (VEGF) by breast cancer cell lines is responsive to endoplasmic reticulum (ER) stress and deprivation of nutrients, including glucose and amino acids. This means that VEGF expression in solid tumors may be increased not just by hypoxia, but also by deficient delivery of other nutrients. We have published three manuscripts on this subject. However, we have been unable to determine the precise mechanism by which VEGF expression is increased in response to glucose and amino acid deprivation of human breast cancer cell lines. We have tested the role of several signal transduction pathways and transcription factors that are part of the unfolded protein response (UPR). This response signals the expression of genes that are upregulated in response to many ER stresses and glucose deprivation. We found that the transcription factor ATF4 plays an important role in VEGF expression of some cells in response to ER stress. However, ATF4 is not necessary for the response to amino acid (glutamine) deprivation and glucose deprivation of breast cancer cell lines. Thus, the mechanism by which deprivation of glucose and amino acids upregulate VEGF expression by breast cancer cells is still unknown.

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Introduction

The BCRP Concept Award helped support work that led to 4 manuscripts (3 published and 1 submitted) and 3 published abstracts. In addition, unpublished data is presented here that documents our attempts to test the role of two transcription factors, ATF6 and ATF4, that are activated in response to nutrient deprivation and UPR activation in the mechanism of VEGF upregulation in response to nutrient stress. Although these studies are not publishable in their current state, they have provide insight that is guiding further mechanistic studies.

Body

We confirmed the hypothesis that VEGF expression is responsive to ER stress and nutrient deprivation. We determined that upregulation was due to transcriptional and post-transcriptional mechanisms. We tested the hypothesis that NF-κB activity was responsible for increased VEGF transcription during glutamine deprivation and found that it was not. (However, upregulation of IL-8 expression in response to glutamine deprivation was NF-κB-dependent.) We tested the role of ATF4 in upregulation of VEGF in response to ER stress and oxidative stress. These studies led to the following publications:

Publications:

1. P.L. Marjon, Y.V. Bobrobnikova and **S.F. Abcouwer**. *Nutrient deprivation and chemical inducers of endoplasmic reticulum stress upregulate the expression of pro-angiogenic factors vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8).* Molecular Cancer 3:4-15, 2004.
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3. Y.V. Bobrobnikova, P.L. Marjon and **S.F. Abcouwer.** *Glutamine deprivation of TSE breast cancer cells causes upregulation of pro-angiogenic factors IL-8 and VEGF: the role of transcription factor NF-kappaB.* Cancer Res. 64:4858-4869, 2004.
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Published Abstracts:

1. **Abcouwer, S.F.**, P.L. Marjon, Y.V. Bobrobnikova, C.N. Roybal, S. Yang and D.L. Vander Jagt. *The role of nutrient stress and ER stress mechanisms in control of pro-angiogenic gene expression.* FASEB J. 17:A1091 (abstract #681.4), 2003.
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In addition to published data, we have tested the role of two transcription factors, ATF6 and ATF4, that are activated in response to nutrient deprivation and UPR activation in the mechanism of VEGF upregulation in response to nutrient stress.

Unpublished data:

Expression of a dominant negative (DN) mutant of ATF6 (ATF6Δ) that does not bind DNA seems to inhibit the response of VEGF mRNA expression to glucose and glutamine deprivation. We constructed adenoviral vectors for the delivery and expression of active wild type ATF6 and a DN mutant ATF6. Human breast carcinoma (TSE) cells infected with two dilutions of these two vectors (1:40 and 1:20) were allowed to express these proteins for 24 h and then treated with control

medium, medium lacking glucose and media lacking glutamine for 18 h. Northern blotting was performed (Figure 1) to determine the effects on expression of VEGF, glucose regulated protein 78 (GRP78) and growth arrest and DNA damage inducible gene 153 (GADD153). Both GRP78 and GADD153 are induced by nutrient deprivation and both are responsive to ATF6. The expression of all three genes seemed to be less in the presence of ATF6Δ, suggesting that ATF6 function may contribute to their expression.

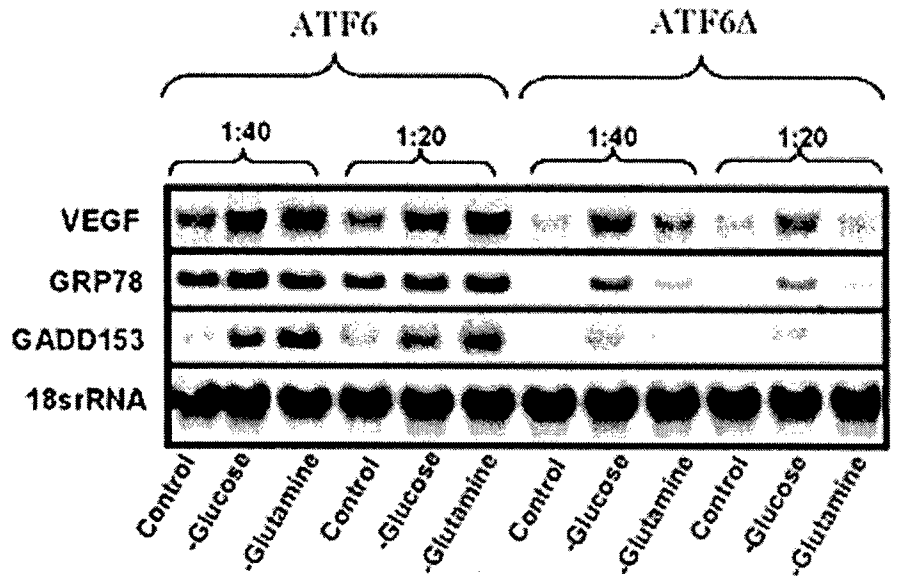

Figure 1. Expression of VEGF, GRP78 and GADD153 mRNAs under nutrient deprivation conditions in the presence of different amounts of ATF6 wt or ATF6 DN mutant (ATF6Δ).

Expression of a DN mutant of ATF6 (here designated ATF6M1) was further tested for the ability inhibit the response of VEGF mRNA expression to glucose and glutamine deprivation. TSE cells were infected with adenoviral vectors expressing active wt ATF6 (ATF6-1-373) or DN ATF6 (ATF6M1) or Empty viral vector and allowed to express for 24 h and then treated with control medium, medium lacking glucose, media lacking glutamine or control medium in an anoxic environment for 18 h. Northern blotting was performed (Figure 2) to determine the effects on expression of VEGF, interleukin-8 (IL-8), GRP78 and GADD153. We recently found that IL-8 expression is responsive to nutrient deprivation by a mechanism that requires NF-κB and AP-1 function. In control conditions, ATF6 function had very little effect on VEGF and IL-8 mRNA expression, although ATF6 DN expression may have reduced VEGF expression slightly. Wt ATF6 expression increased GRP78 and GADD153 expression, whereas ATF6 DN expression did not. Under glutamine-starved conditions, VEGF and IL-8 mRNA expression was greatly increased in Empty vector infected cells. Expression of wt ATF6 did not increase this effect, but expression of DN ATF6 did seem to decrease this effect slightly. Expression of GRP78 and GADD153 in glutamine-starved cells was markedly increased by wt ATF6, but not effected by the DN ATF6. In glucose-deprived cells the results were similar, but not identical. Expression of wt ATF6 did not markedly increase the expression of any of these genes. Expression of DN ATF6 did seem to decrease the effect of glucose deprivation on VEGF expression slightly. Expression of GRP78 and GADD153 in glucose-starved cells was decreased by the DN ATF6 expression. ATF6 function did not affect the induction of VEGF expression by anoxia.

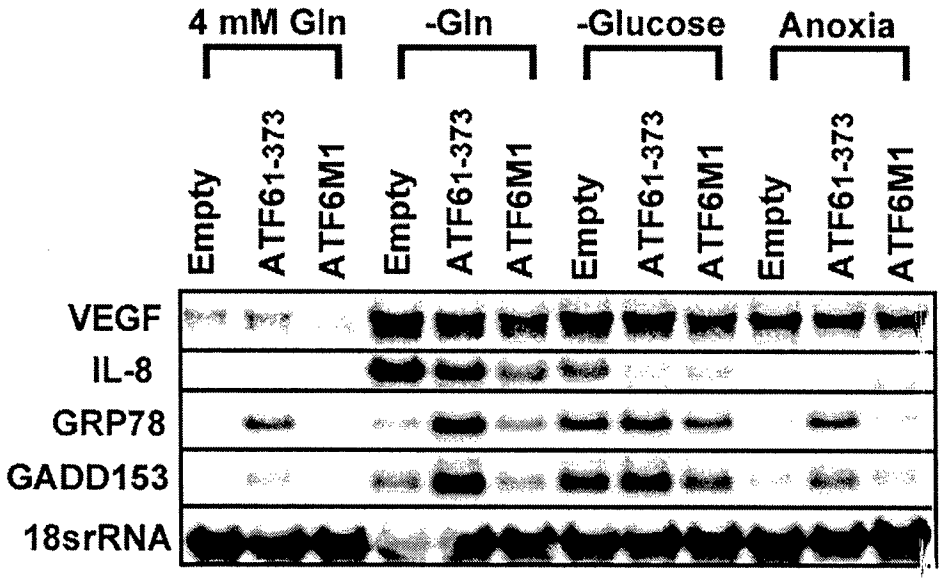

Figure 2. Expression of VEGF, IL-8, GRP78 and GADD153 under nutrient/oxygen deprivation conditions in the presence of ATF6 wt or ATF6 DN proteins.

The results shown in Figures 1 and 2 led us to conclude that ATF6 has only a minor role in the control of VEGF expression in response to nutrient deprivation. However, because the DN ATF6 mutant did not dramatically reduce GRP78 and GADD153 expression, we looked for another way to inhibit ATF6 function. AEBSF is a protease inhibitor that was recently shown to inhibit site-1 protease (S1P). The activity of this protease is required for activation of ATF6, by cleavage of ATF6-p90 to ATF6-p50 in the Golgi. AEBSF was shown to completely block ATF6 cleavage and activation. Figure 3 shows the effects of AEBSF treatment on the expression of VEGF, IL-8 GRP78 and GADD153 mRNA expression in control cells and cells subjected to nutrient deprivation, ER stress (tunicamycin treatment) and extended anoxia. Surprisingly, AEBSF treatment slightly increased VEGF, GRP78 and GADD153 mRNA expression in unstressed control cells. AEBSF inhibited VEGF, IL-8 and GRP78 mRNA expression in glutamine-starved cells. This is true for glucose-starved cells as well. However, AEBSF did not effect GADD153 expression during glutamine deprivation, but did decrease GADD153 expression during glucose deprivation. AEBSF had very little affect on tunicamycin-treated cells. Cells were also subjected to 24 h of anoxia (which, compared to 18 h, causes increased VEGF expression and upregulates IL-8, GRP78 and GADD153). AEBSF did not inhibit VEGF mRNA expression in anoxic cells, but it did inhibit GRP78 and GADD153 mRNA expression during anoxia.

The results shown in Figure 3 are hard to interpret. They may indicate that ATF6 processing by S1P is necessary for VEGF upregulation in response to nutrient deprivation. However, there is a possibility that another S1P substrate is actually necessary. In addition, AEBSF also inhibits NADPH oxidase activity. Thus, it is possible that this inhibitor exerts an effect on VEGF expression by blocking oxidative signaling by NADPH oxidase.

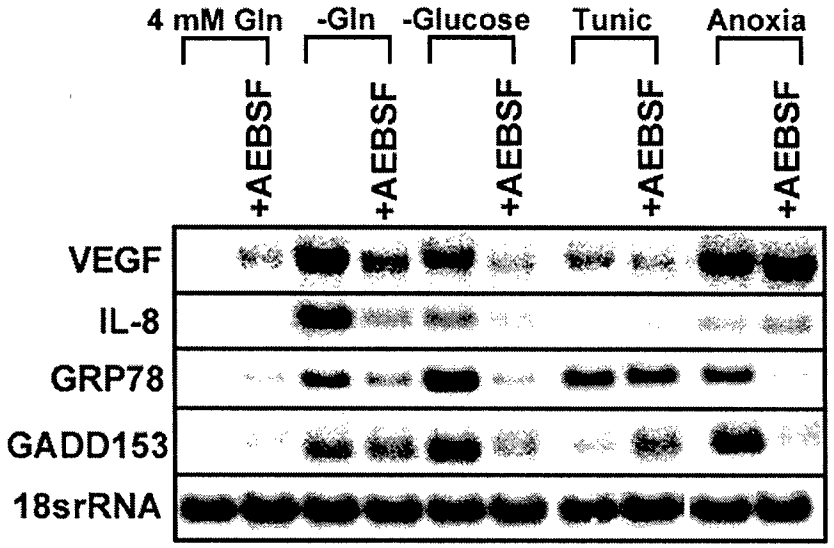

Figure 3. Effect of site-1 protease (S1P) inhibitor AEBSF on the expression of VEGF, IL-8, GRP78, and GADD153 mRNA under nutrient deprivation ER stress (tunicamycin treatment, Tunic) and anoxia.

We next tested the necessity of ATF4 function on VEGF induction in response to nutrient deprivation. We constructed adenoviral vectors for the delivery and expression of active wild type ATF4 and a DN mutant of ATF4 (ATF4ΔRK) that does not bind DNA Human breast carcinoma (TSE) cells infected with these two vectors, were allowed to express these proteins for 24 h and then treated with control medium, medium lacking glucose and media lacking glutamine for 18 h. Northern blotting was performed (Figure 4) to determine the effects on expression of VEGF, Il-8, GRP78 and GADD153 mRNA. Expression of wt ATF4 slightly increased the expression of VEGF mRNA in control cells, but did not markedly accentuate the expression of VEGF in stressed cells. Expression of the DN ATF4 decreased VEGF induction only slightly. This effect was only considerable in tunicamycin and brefeldin A treated cells (both treatments that cause ER stress). Thus, ATF4 function did not seem to be key to VEGF induction by nutrient deprivation.

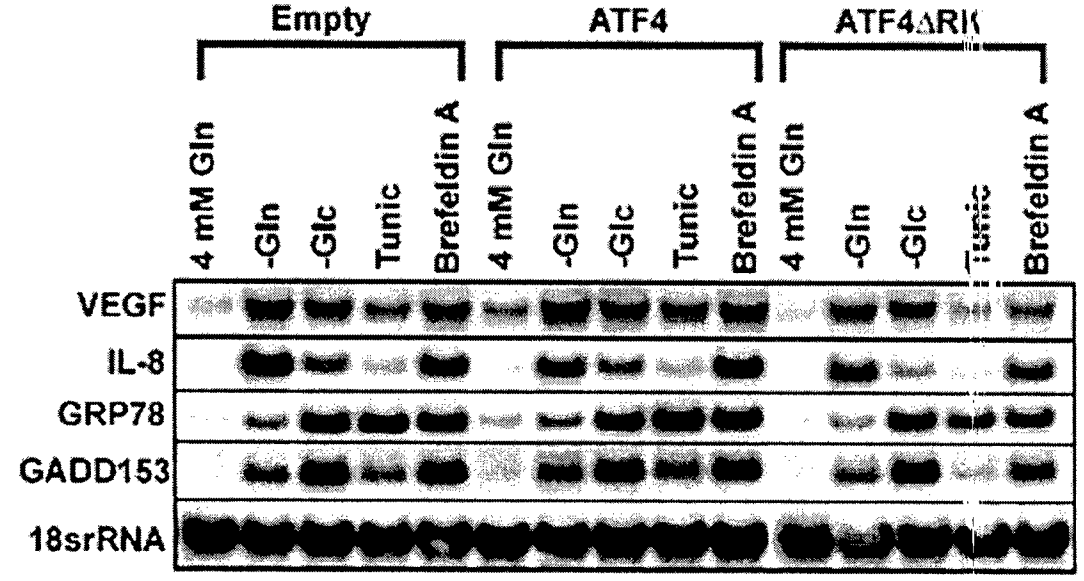

Figure 4. Effect of wt ATF4 and a DN mutant ATF4 (ATF4ΔRK) on the expression of VEGF, IL-8, GRP78 and GADD153 mRNA under nutrient-deprived and ER stress conditions.

In conclusion, we have no definitive data to suggest that ATF6 or ATF4 function is necessary for upregulation of VEGF expression in response to nutrient deprivation and ER stress. Our most intriguing finding is that AEBSF effectively blocks VEGF upregulation. We are currently following up this result by expressing ATF6-1-373 and then treating cells with

AEBSF. The ATF6-1-373 corresponds to ATF6p50, and therefore does not require cleavage by S1P. We are also planning to examining the effects of inhibiting NADPH oxidase by means other than AEBSF, to test if this function causes the effects of AEBSF on VEGF expression.

Key research Accomplishments

- Determined that VEGF expression is upregulated by ER stress and nutrient deprivation.
- Determined that upregulation was both transcriptional and post-transcriptional.
- Tested the necessity of NF-κB transcription factor in upregulation of VEGF.
- Tested the role of ATF6 in upregulation of VEGF.
- Tested the role of ATF4 in upregulation of VEGF.
- Found that AEBSF inhibits upregulation of VEGF.

Reportable outcomes

Publications:

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Conclusions:

VEGF expression is responsive to ER stress and nutrient deprivation. The transcription factor ATF4 plays a role in this regulation under some circumstances. However, the signal transduction mechanisms and transcription factors that are responsible for increased VEGF transcription by breast cancer cells that are subjected to nutrient deprivation have not been determined. The almost complete abrogation of VEGF induction by AEBSF provides starting place to determine a mechanism. Two hypotheses are being tested: 1) AEBSF inhibits VEGF expression by blocking the activation of an unknown transcription factor that is processed by S1P. 2) AEBSF inhibits VEGF expression by inhibiting NADPH oxidase activity.

[CANCER RESEARCH 64, 4858–4869, July 15, 2004]

Expression of Angiogenic Factors Vascular Endothelial Growth Factor and Interleukin-8/CXCL8 Is Highly Responsive to Ambient Glutamine Availability: Role of Nuclear Factor-κB and Activating Protein-1

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[1]Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, and [2]University of New Mexico Cancer Research and Treatment Center, Albuquerque, New Mexico

ABSTRACT

Vascular endothelial growth factor (VEGF) and interleukin-8/CXCL8 (IL-8) are prominent pro-angiogenic and pro-metastatic proteins that represent negative prognostic factors in many types of cancer. Hypoxia is thought to be the primary environmental cause of VEGF and IL-8 expression in solid tumors. We hypothesized that a lack of nutrients other than oxygen could stimulate the expression of these factors and previously demonstrated that expression of VEGF and IL-8 is responsive to amino acid deprivation. In the present study, we examined the effect of glutamine availability on the expression of these factors as well as the role of transcription factors NFκB and activating protein-1 (AP-1) in the response of TSE human breast carcinoma cells to glutamine deprivation. VEGF and IL-8 secretion and mRNA levels were dramatically induced by glutamine deprivation. mRNA stabilization contributed to this response. Glutamine deprivation increased NFκB (p65/p50) and AP-1 (Fra-1/c-Jun+JunD) DNA-binding activities. Blocking NFκB and AP-1 activation with curcumin as well as expression of dominant inhibitors, inhibitor of nuclear factor-κB (IκB) super repressor (IκBM), and a mutant form of c-Fos (A-Fos) demonstrated that the activation of NFκB and AP-1 transcription factors was necessary for the induction of IL-8 expression but dispensable for the induction of VEGF expression. A macro-array containing 111 NFκB target genes identified a total of 17 that were up-regulated 2-fold or more in response to glutamine deprivation. These included growth regulated oncogene α (*GROα/GRO1/CXCL1*), another neutrophil chemoattractant implicated in tumor angiogenesis and metastasis.

INTRODUCTION

Angiogenesis is regulated by a number of positively and negatively acting effectors. Vascular endothelial growth factor A (VEGF), also known as vascular permeability factor, is a prominent pro-angiogenic and tumor growth-promoting hormone expressed in a wide range of tumor cells. Expression of VEGF is often obligatory for tumor angiogenesis, thus inhibition of VEGF expression or function has been fervently pursued as a cancer treatment (1). It is well established that VEGF expression is induced by hypoxia through transcriptional activation and mRNA stabilization (2–4). Thus, solid tumors respond to low oxygen tension by increasing VEGF expression and thereby promoting increased oxygen delivery through angiogenesis.

Interleukin-8 (IL-8, CXCL8) belongs to the ELR (Glu-Leu-Arg) motif-positive (ELR+) CXC subclass of chemokines and is the most potent lymphocyte chemoattractant in this group. IL-8 has recently been shown to contribute to cancer progression by acting as a mitogenic, angiogenic, and motogenic factor (reviewed in Ref. 5). The role of IL-8 in breast cancer was observed in several studies in which its expression has been strongly correlated with metastatic phenotype (6–8). However, the mechanisms by which IL-8 supports tumor growth, angiogenesis, and metastasis are not precisely understood. IL-8 has also been demonstrated to induce migratory responses of endothelial cells through interaction with its cognate G protein-coupled receptors CXCR1 and CXCR2 (9, 10). Thus, IL-8 may promote the breakdown of extracellular matrix components within solid tumors through its ability to attract protease-laden neutrophils (11) and may stimulate nascent blood vessel growth and attraction by acting on endothelial cells (12). IL-8 may trigger tumor egress by directly inducing the motility of tumor cells that possess CXCR1 or CXCR2 receptors (7, 13). Extrinsic factors and intrinsic genetic influences control the expression of IL-8 by cancer cells. Like VEGF, IL-8 is responsive to tumor-related environmental factors, including hypoxia (14).

The tumor microenvironment is also characterized by insufficient vascular delivery of nutrients such as glucose and amino acids. This is exacerbated by the fact that cancer cells often exhibit greatly increased rates of glucose utilization (via an aerobic glycolysis) and glutamine consumption. For example, tumor tissues exhibit low interstitial glucose concentrations (15, 16). In 1995, Keshet's group first demonstrated that VEGF expression was increased in response to glucose deprivation (17, 18). In addition, we have shown that xenografted tumors formed from a highly glutamine-dependent human breast carcinoma cell line experience reduced intratumoral glutamine contents as they grow in size (19). It seems logical that, in analogy to oxygen deprivation, solid tumors would respond to glutamine deprivation by initiating angiogenesis. Alternatively, tumor cells could escape a nutrient-poor environment by increasing their ability to metastasize to a secondary tumor site. Thus, we hypothesized that deprivation of glutamine could affect expression of pro-angiogenic and pro-metastatic factors. We recently demonstrated that the expression of VEGF and IL-8 was induced by glucose and amino acid deprivation of breast carcinoma cell lines (20). This was the first study to demonstrate that IL-8 expression is responsive to nutrient deprivation and only the second to demonstrate that VEGF expression is responsive to amino acid deprivation, the first being our study using a human retinal pigmented epithelial cell line (21). However, the mechanisms and mediators of these inductions were not determined. It seemed that transcription factors nuclear factor-κB (NFκB) and activating protein-1 (AP-1) were likely candidates for this mechanism, because they each affect the transcription of *VEGF* and *IL-8* genes. *IL-8* transcription is highly responsive to activation of NFκB and AP-1 (22, 23). IL-8 expression in melanoma and pancreatic cancer cell lines has been shown to directly correlate with NFκB activity (24, 25). NFκB has also been implicated in the control of *VEGF* transcription (26, 27), and VEGF and IL-8 seem to be coexpressed by several

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Requests for reprints: Steve F. Abcouwer, University of New Mexico, School of Medicine, Biochemistry and Molecular Biology, MSC08-4670, 915 Camino de Salud NE, Albuquerque, NM 87131. E-mail: sabcouwer@salud.unm.edu.

tumor types (28–32). Inhibition of NFκB activity reduced IL-8 and VEGF expression as well as tumorigenicity and angiogenesis of prostate and ovarian cancer cells (33, 34). AP-1 has been shown to act synergistically with NFκB to promote *IL-8* transcription (23, 35, 36) and to play a role in IL-8 expression in response to hypoxia (35, 37). AP-1 has also been shown to play a positive role in the induction of VEGF expression by hypoxia, transforming growth factor-β, and acidosis (38–41).

In the present work, the expression of VEGF and IL-8 in response to glutamine deprivation was studied in detail using a highly glutamine-dependent human breast carcinoma cell line (TSE cells). We hypothesized that glutamine deprivation of TSE cells increases *VEGF* and *IL-8* transcription through NFκB and AP-1 activation. However, when this study was initiated, there was no indication in the literature that either NFκB or AP-1 activity is affected by nutrient availability. Very recently, Jiang *et al.* (42) showed that NFκB is activated in response to leucine deprivation. To test this hypothesis, the effects of glutamine starvation on VEGF and IL-8 secretion, mRNA levels, and mRNA turnover, as well as activation of NFκB and AP-1 DNA-binding activities were examined. Glutamine deprivation caused marked activation of AP-1 and NFκB, therefore pharmacological and genetic means were used to inhibit NFκB and AP-1 activities and to determine the effects on gene expression. Curcumin, a component of the curry spice turmeric that is now in clinical trials as a cancer preventative agent, was used to inhibit activation of NFκB and AP-1. In addition, recombinant adenoviruses expressing dominant-negative repressors of NFκB [inhibitor of nuclear factor-κB (IκB) super repressor, IκBM] or AP-1 (A-Fos) functions were used to test the necessity of these factors. Our results indicate that NFκB and AP-1 activation was necessary for the induction of IL-8 transcription in response to glutamine deprivation. In contrast, these transcription factors were not necessary for the induction of VEGF expression. Finally, a cDNA macro-array containing 111 NFκB target genes identified 17 genes that were up-regulated in response to glutamine deprivation (including *IL-8*). Up-regulated genes also included the neutrophil chemoattractant growth-regulated oncogene α (*GROα/GRO1/CXCL1*), which may play a role in tumor progression similar to that of IL-8.

MATERIALS AND METHODS

Materials. Tissue culture media and medium supplements were purchased from Invitrogen Life Technologies, Inc. (Grand Island, NY). All chemicals, drugs, and reagents were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Chemicals (St. Louis, MO) except for WST-1 cell proliferation reagent, which was purchased from Roche Applied Science (Indianapolis, IN). Human breast adenocarcinoma TSE cells were provided by Dr. Simon Powell (Radiation Oncology, Massachusetts General Hospital, Boston, MA).

Cell Culture. TSE cells were cultured in DMEM (high glucose and no glutamine formulation) supplemented with 4 mM L-glutamine, 10% (v/v) fetal bovine serum, 10 μg/ml bovine insulin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C, in a humidified atmosphere of 5% CO_2 and 95% air. For Northern blotting and ELISA experiments, cells were plated in 60-cm^2 tissue culture dishes, grown to confluence, and refed with fresh media 1 day before being rinsed twice with Dulbecco's PBS and fed with fresh media (10 ml/plate) containing drugs or other treatments as described in figure legends. For glutamine deprivation experiments, glutamine-free media (DMEM lacking glutamine and supplemented with 10% (v/v) dialyzed fetal bovine serum) was used as described in figure legends. For DNA-binding activity ELISA-like assays, cells were plated in 150-cm^2 plates, grown to confluence, and refed with fresh media 1 day before being rinsed twice with Dulbecco's PBS and fed with fresh media (30 ml/plate) containing drugs or other treatments as described in figure legends.

Plasmids. The pCMV-IκBαM plasmid encoding NFκB dominant-negative mutant IκB super repressor was purchased from Clontech (Palo Alto, CA). The CMV500–8584hep-fosLZ(MO) plasmid-encoding c-Fos dominant-negative mutant A-Fos (43) was kindly provided by Dr. Charles Vinson (Laboratory of Biochemistry, National Cancer Institute, NIH).

Northern Blotting. Cells were harvested using RNA-STAT60 reagent (TelTest, Friendswood, TX). The total cellular RNA was isolated using one-step acid-phenol guanidinium procedure (44) with subsequent acid-phenol, phenol/chloroform/isoamyl alcohol, and chloroform extractions and ethanol precipitation in the presence of sodium acetate. Equal amounts of total RNA (10 μg) were fractionated by electrophoresis, transferred to nylon membrane (Micron Separations, Inc., Westborough, MA) by capillary action, and UV-light cross-linked to the membrane at 120,000 μJ/cm^2. Membrane was incubated at 65°C for 5 h in blocking solution with final concentrations of 5× saline-sodium phosphate-EDTA, 7.5× Denhardt's solution, and 0.5% SDS. Northern blotting was performed using cDNAs corresponding to human *VEGF* (dbEST189750), *glucose-regulated protein 78* [*GRP78* (dbEST107273)], *IL-8* (dbEST6044688), *growth arrest and DNA damage-inducible gene 153* [*GADD153* (dbEST298470)], *GADD45* (dbEST602435), and *18S rRNA* as templates to generate ^{32}P-labeled probes with a random-primer labeling kit (Amersham Biosciences, Piscataway, NJ). A rat *18S rRNA* cDNA template was reverse transcription-PCR generated from total rat kidney RNA using R18F2 sense (5′-GCTACCACATCCAAGGAAGGC-3′) and R18B1 antisense (5′-CCCGTGTTGAGTCAAATTAAGCC-3′) primers. Northern blots were quantified using a STORM PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Fold inductions were obtained by dividing integrated band intensity volumes for experimental samples by that of control (untreated, time zero, or complete media) samples.

ELISA. ELISA assays were performed using commercial VEGF and IL-8 ELISA kits from R&D Systems (Minneapolis, MN). Conditioned media was collected from 60-cm^2 plates, aliquoted, and stored (1–4 months) frozen until being assayed. Samples were diluted 4–20-fold in deionized water before assaying. Assays were performed in triplicate, and readings were compared with standard curves obtained with human recombinant $VEGF_{165}$, provided with the kit.

mRNA Stability Analysis. TSE cells were incubated for 6 h in glutamine-free medium to induce VEGF and IL-8 mRNA levels and then transferred to either fresh glutamine-free medium or amino acid-complete medium, both containing 5 μM RNA synthesis inhibitor actinomycin D. Total cellular RNA was isolated at time points indicated in figure legend and subjected to Northern blotting. The data were quantified using phosphorimagery and expressed as a fraction of the value obtained for time 0 (6 h of glutamine deprivation). To calculate mRNA half-lives ($t_{1/2}$), the data were linearized by plotting the natural logarithm of fractional response [fractional response = $(R_t - R_0)/(R_{ss} - R_0)$], where R_t is the relative amount of mRNA at the time point t, R_0 is the relative amount of mRNA after 24 h of treatment with actinomycin D, and R_{ss} is the steady-state relative amount of mRNA (after 6 h of glutamine deprivation) *versus* time. The slope values obtained from linear regression were used to calculate the $t_{1/2}$ ($t_{1/2}$ = ln2/slope).

DNA-Binding Activity Analysis. Nuclear extracts were obtained from glutamine-starved and glutamine-fed TSE cells using TransFactor Extraction kit (Clontech) according to the manufacturer's protocol. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). DNA-binding activity for transcription factor NFκB RelA/p65 in the nuclear extracts was quantitated employing ELISA-like Mercury TransFactor kit (Clontech) or TransAM NFκB kit (Active Motif, Carlsbad, CA) according to the manufacturers' protocol. Electrophoretic mobility shift assays (EMSAs) were performed using Gel Shift Assay Systems (Promega, Madison, WI) according to manufacturer's protocol. The sequence of the double-stranded DNA (dsDNA) fragment containing NFκB-binding element was 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and for AP-1 consensus was 5′-CGCTTGATGAGTCAGCCGGAA-3′ (oligonucleotides provided with the kit). In the binding reactions, ^{32}P-labeled DNA fragments were incubated with 10 μg of nuclear extract. Binding assays were performed at room temperature for 20 min, and the DNA-protein complexes were separated by electrophoresis on 5% Tris-borate EDTA PAGE gel and visualized using STORM PhosphorImager and Image Quant software (Molecular Dynamics, Sunnyvale, CA). For NFκB supershift studies, 1.5 μg of anti-NFκB p65-specific polyclonal antibody (Zymed Laboratories, South San Francisco, CA) or 2 μg of anti-NFκB p50-specific polyclonal antibody (sc-114; Santa Cruz Biotechnology, Santa Cruz, CA) were included in the binding mixture 10 min before loading reactions on

the gel. For AP-1 supershift studies, 4 μg of each rabbit polyclonal anti-c-Fos (K-25), anti-c-Fos (H-125, recognizing an epitope common to c-Fos, FosB, Fra-1, and Fra-2), anti-Fra-1 (R-20), anti-Fra-2 (L-15), goat polyclonal anti-FosB (102; Santa Cruz Biotechnology), rabbit polyclonal anti-JunD (Ab425; Abcam, Cambridge, MA), or rabbit polyclonal anti-c-Jun (Cell Signaling, Beverly, MA) antibody were preincubated with 10 μg of nuclear extract at 4°C for 1 h before addition of the ^{32}P-labeled dsDNA probe.

Adenoviruses Construction and Production. IκBM and A-Fos were expressed using the AdEasy adenoviral vector system (Ref. 45; kindly provided by Bert Vogelstein at Howard Hughes Institute, Johns Hopkins University). The A-Fos sense primer (5′-CAAACAACAGCGGCCGCCCACCATGGACTACAAG-3′) and the A-Fos antisense primer (5′-CCCTCTAGAAGCTTGAATTAA-3′), and the IκBM sense primer (5′-TATTCATCTGCGGCCGCAGCTTATGTTCCAGGCG-3′) and the IκBM antisense primer (5′-AAATCGTTTAAGCTTTCATAACGTCAGACGCTG-3′) were used to introduce *Not*I and *Hin*dIII restriction sites (underlined) at the 5′ and 3′ termini, respectively, of the A-Fos and IκBM cDNAs. PCR products were cloned into pAdTrack-CMV adenoviral shuttle vector using *Not*I and *Hin*dIII restriction sites. The shuttle vectors were then linearized with the restriction enzyme *Pme*I and electroporated into DY329 electro-competent cells along with the adenoviral backbone plasmid pAdEasy-1. Clones containing recombinant plasmids pAE-IκBM and pAE-AFos, formed by homologous recombination, were subsequently selected for kanamycin resistance and identified by restriction analysis. Finally, recombinant adenoviral vectors were generated by transfecting the 293 packaging cell line with *Pac*I-linearized pAE-IκBM and pAE-AFos. Successful transfection and viral production were followed with the aid of fluorescent microscopy because the pAdTrack vector contains an enhanced green fluorescent protein expression cassette that is incorporated into the viral vector. Transfected cultures were maintained until the percentage of cells exhibiting green fluorescence approached 100%. Viruses were obtained by freeze/thaw lysis (four times) of the cells in PBS, followed by clarification of the lysates by centrifugation. The final high-titer viral stock in PBS was prepared by transfecting ten 150-mm diameter plates of 293 cells. After freeze/thaw lysis (four times) of the cells in PBS, cellular debris was pelleted by centrifugation, and the raw supernatant was used at 1:20 in serum-free media to infect target cells.

NFκB Target Gene Analysis. The expression of NFκB target genes in glutamine-starved and glutamine-fed TSE cells was analyzed using cDNA-cDNA hybridization-based TranSignal NFκB Target Gene Array kit (Panomics, Redwood City, CA). Total RNAs from glutamine-fed and -starved cells were isolated as described above and then used to produce biotin-labeled cDNA probes using the manufacturer's protocol and reagents. The arrays were hybridized, washed, and developed per the manufacturer's instructions. The signal was detected using streptavidin-horseradish peroxidase conjugate and a mixture of luminol enhancer and peroxide solution as substrate. The membranes were then scanned and viewed using a MultiGenius Bioimaging System (Cambridge, United Kingdom).

RESULTS

Effect of Ambient Glutamine Deprivation on VEGF and IL-8 Expression. To determine the effect of ambient glutamine limitation on VEGF and IL-8 protein secretion by TSE cells, accumulation of VEGF and IL-8 proteins in the culture media was analyzed by ELISA of samples collected after various times of complete glutamine deprivation (Fig. 1*A*). Secreted VEGF protein increased continuously during 4–48 h of glutamine starvation, reaching 15.3 ± 0.2 ng/ml concentration in the media compared with 0.53 ± 0.04 ng/ml in media of glutamine-fed cells at 22 h. IL-8 protein also continuously accumulated over 48 h of glutamine deprivation, reaching a concentration of 68 ± 9.6 ng/ml compared with 0.88 ± 0.25 ng/ml in media of glutamine-fed cells at 22 h. Thus, TSE cells responded to glutamine deprivation by secreting relatively large amounts of VEGF and IL-8 proteins. No cell death was visibly apparent when confluent cultures of TSE cells were glutamine starved for as long as 48 or 72 h. The ability of confluent TSE cell cultures to survive glutamine starvation with no appreciable cell loss was confirmed by measuring reduction of the cell proliferation reagent WST-1 to formazan dye in replicate cultures subjected to total glutamine deprivation for various times up to 48 h (data not shown).

The sensitivity of VEGF and IL-8 mRNA expression to ambient glutamine concentration was examined using confluent cultures of TSE breast carcinoma cells. Because amino acid deprivation causes cellular stress resulting in the induction of expression of endoplasmic reticulum (ER) chaperone protein GRP78 and transcription factor GADD153, the mRNA levels of these genes were used as indicators of the activation of ER stress and nutrient stress response pathways. Cell cultures were incubated with media containing serial dilutions of glutamine from 4 to 0.06 mM, as well as no glutamine, for 8 h, and then relative mRNA levels were assayed by Northern blotting (Fig. 1*B*). As initial ambient glutamine concentrations fell below physiological levels (0.6 mM), VEGF mRNA levels increased. The induction was 2-fold at 0.25 mM glutamine and continued to increase with decreasing glutamine concentration, reaching a 6-fold induction in the absence of glutamine. The IL-8 mRNA levels showed a similar sensitivity to ambient glutamine. IL-8 mRNA induction was 4-fold at 0.25 mM glutamine and continued to increase with decreasing glutamine, exhibiting a maximum induction of approximately 50-fold when glutamine was absent. The expression patterns for GADD153 and GRP78 mRNAs were qualitatively similar to those of VEGF and IL-8. In fact, the induction of GADD153 expression was nearly identical to that of VEGF. However, GRP78 mRNA levels reached a local maximum (1.9-fold induction) at 1 mM glutamine while demonstrating an absolute maximum of 3-fold induction in the absence of glutamine. Thus, the expression of VEGF and IL-8 mRNAs was increased as initial ambient glutamine concentrations fell below physiological levels, and these responses coincided with the expression of ER stress and nutrient stress-responsive genes *GRP78* and *GADD153*.

The temporal responses of VEGF and IL-8 mRNA levels to complete glutamine deprivation were then determined and compared with those for GADD153 and GRP78 mRNAs (Fig. 1*C*). VEGF mRNA levels increased 3-fold within 4 h of glutamine deprivation and continued to rise with time, reaching a steady-state level of 7.5-fold induction at 16 h. The accumulation of IL-8 mRNA was more rapid, resulting in a 4-fold induction after only 2 h of glutamine starvation. IL-8 mRNA levels increased to a maximal 140-fold by 8 h and then decreased to 55-fold above the basal level after 48 h of glutamine deprivation. The expression pattern for GADD153 was quantitatively similar to that for VEGF. However, GADD153 mRNA levels increased 8-fold by 8 h and then slightly decreased down to 5.5-fold induction over the next 40 h. The expression pattern for GRP78 was qualitatively similar to VEGF. GRP78 mRNA levels demonstrated a slow rise with a 1.4-fold induction at 4 h, reaching a steady-state level of 3-fold induction at 16 h. Thus, VEGF mRNA levels showed a sustained induction in response to glutamine starvation that was temporally similar but to greater than that of GRP78 mRNA. IL-8 mRNA levels showed a very large sustained induction that was temporally similar to but greater than that of GADD153.

The Effect of Glutamine on VEGF and IL-8 mRNA Turnover. To evaluate the relative contributions of transcriptional and posttranscriptional mechanisms in up-regulating VEGF and IL-8 mRNAs during glutamine deprivation, the decay rates of VEGF and IL-8 mRNAs were analyzed in glutamine-deprived and glutamine-fed TSE cells. GADD45 mRNA decay was used as a positive control because it was previously demonstrated to be stabilized by glutamine deprivation (46). To determine the effect of glutamine on mRNA decay rates, cultures of TSE cells were first starved of glutamine to raise mRNA levels, then treated with actinomycin D to stop transcription, and incubated in the absence or presence of ambient glutamine for various times when mRNA levels were assayed (Fig. 2*A*). Decay

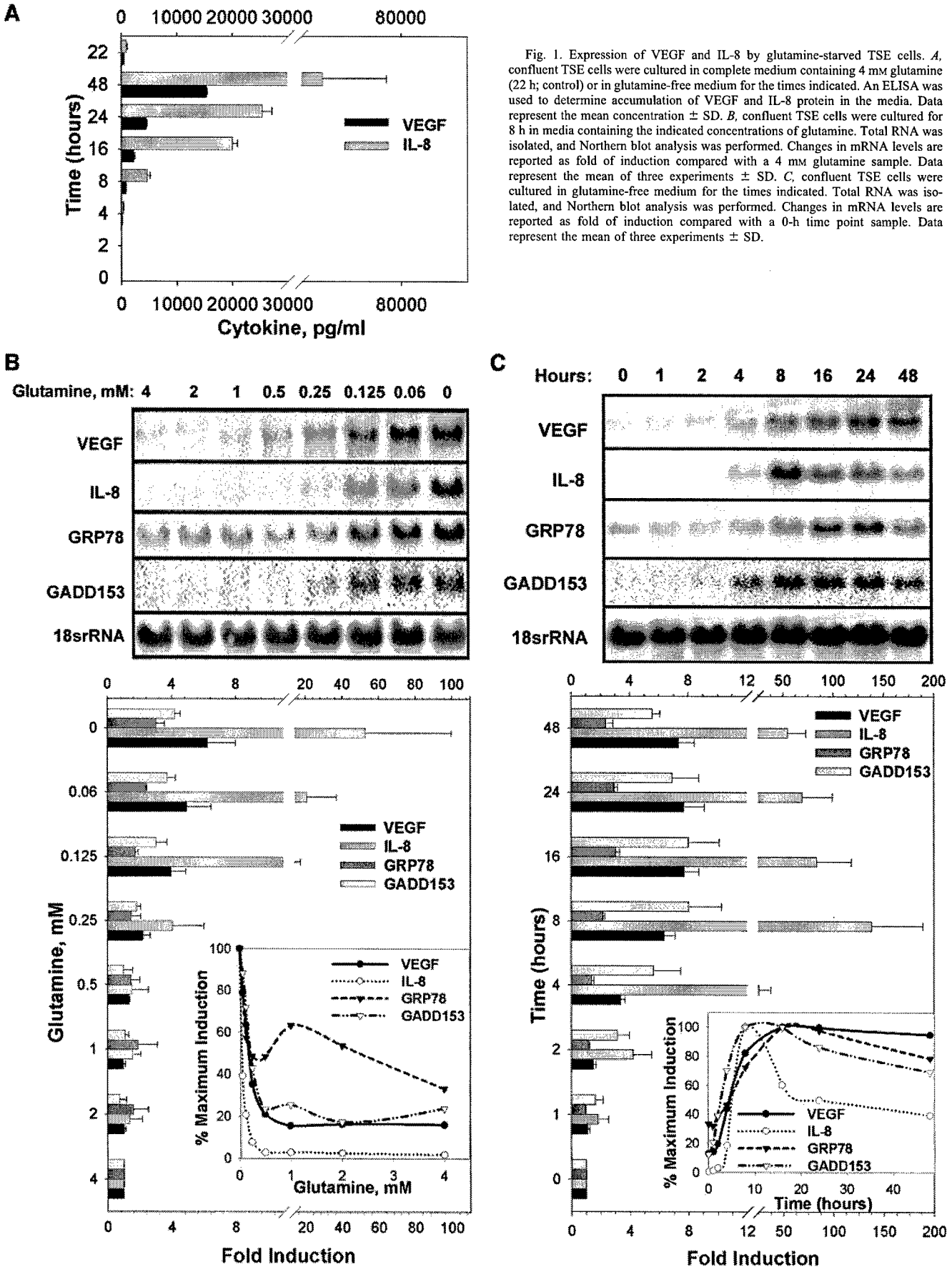


Fig. 1. Expression of VEGF and IL-8 by glutamine-starved TSE cells. *A*, confluent TSE cells were cultured in complete medium containing 4 mM glutamine (22 h; control) or in glutamine-free medium for the times indicated. An ELISA was used to determine accumulation of VEGF and IL-8 protein in the media. Data represent the mean concentration ± SD. *B*, confluent TSE cells were cultured for 8 h in media containing the indicated concentrations of glutamine. Total RNA was isolated, and Northern blot analysis was performed. Changes in mRNA levels are reported as fold of induction compared with a 4 mM glutamine sample. Data represent the mean of three experiments ± SD. *C*, confluent TSE cells were cultured in glutamine-free medium for the times indicated. Total RNA was isolated, and Northern blot analysis was performed. Changes in mRNA levels are reported as fold of induction compared with a 0-h time point sample. Data represent the mean of three experiments ± SD.

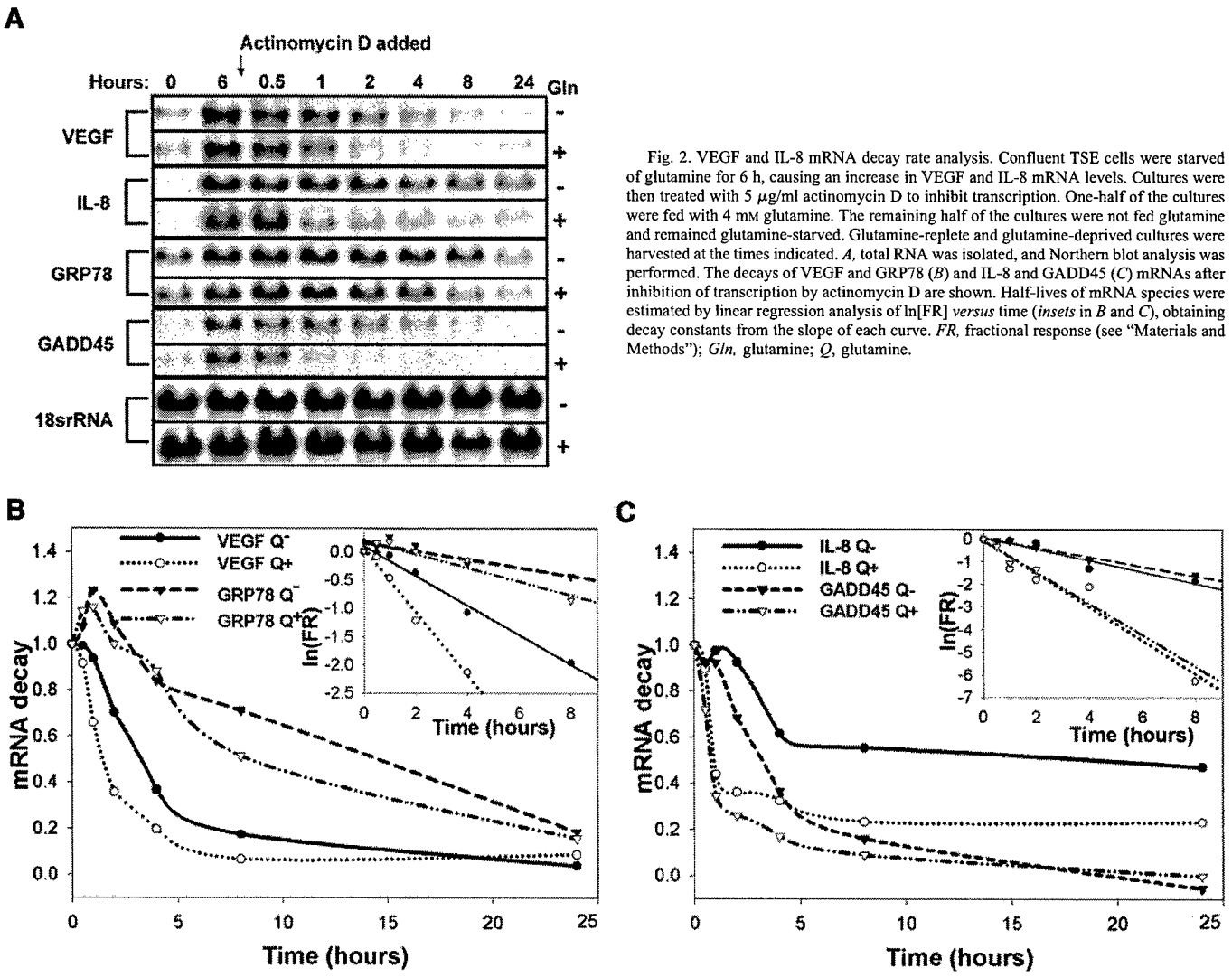


Fig. 2. VEGF and IL-8 mRNA decay rate analysis. Confluent TSE cells were starved of glutamine for 6 h, causing an increase in VEGF and IL-8 mRNA levels. Cultures were then treated with 5 μg/ml actinomycin D to inhibit transcription. One-half of the cultures were fed with 4 mM glutamine. The remaining half of the cultures were not fed glutamine and remained glutamine-starved. Glutamine-replete and glutamine-deprived cultures were harvested at the times indicated. *A*, total RNA was isolated, and Northern blot analysis was performed. The decays of VEGF and GRP78 (*B*) and IL-8 and GADD45 (*C*) mRNAs after inhibition of transcription by actinomycin D are shown. Half-lives of mRNA species were estimated by linear regression analysis of ln[FR] *versus* time (*insets* in *B* and *C*), obtaining decay constants from the slope of each curve. *FR*, fractional response (see "Materials and Methods"); *Gln*, glutamine; *Q*, glutamine.

curves were fitted to a first order kinetic model to obtain decay constants and calculate each mRNA half-life ($t_{1/2}$; Fig. 2, *B* and *C*). The presence of glutamine accelerated VEGF mRNA decay. VEGF mRNA exhibited a $t_{1/2}$ of 2.6 h in continuously glutamine-starved cells and a $t_{1/2}$ of 1.2 h in glutamine-fed cells. This represents a 2.2-fold decrease in turnover rate caused by glutamine deprivation. This magnitude of VEGF mRNA half-lives and the effect of glutamine deprivation is remarkably similar to the values obtained in other cell systems subjected to hypoxia, which also caused a 2–3-fold increase in VEGF mRNA stability (47). The $t_{1/2}$ value for IL-8 mRNA was raised 3.0-fold in the absence of glutamine, with a 2.8 h $t_{1/2}$ compared with 0.93 h $t_{1/2}$ in glutamine-fed cells. The stability of GRP78 mRNA was increased by 1.7-fold in glutamine-starved TSE cells ($t_{1/2}$ of 9.5 h *versus* 5.6 h). The stability of GADD145 mRNA was increased 3.0-fold by glutamine starvation ($t_{1/2}$ of 3.0 h *versus* 1.0 h) when compared with glutamine-fed TSE cells. Thus VEGF and IL-8 mRNA turnover was affected by ambient glutamine. However, the magnitudes of the effects were much less than the observed increases in steady-state VEGF and IL-8 mRNA levels caused by glutamine starvation. This is particularly true for IL-8 mRNA levels, which were increased by up to 140-fold. Therefore, glutamine deprivation must affect VEGF and IL-8 expression by increasing transcription rates as well as by increasing mRNA stability.

Role of NFκB and AP-1 in Induction of VEGF and IL-8 Expression in Response to Glutamine Deprivation. *VEGF* and *IL-8* transcription can be influenced by NFκB and AP-1, however, the effect of glutamine deprivation on the activity of these transcription factors is not known. Therefore, we first tested the effect of glutamine deprivation on the DNA-binding activity for the NFκB and AP-1 complex components. The EMSAs with radiolabeled dsDNAs containing NFκB- and AP-1-binding sites demonstrated that complete glutamine deprivation of TSE cells for 8 h caused NFκB and AP-1 DNA-binding activity in nuclear extracts to increase, resulting in the shift of radiolabeled dsDNA probes (Fig. 3, *A* and *B*). When a polyclonal antibody against the NFκB p65 subunit, the NFκB p50 subunit, or both was included in the binding reaction with the κB-binding site, a supershift was observed, confirming the presence of these NFκB subunits in the complex (Fig. 3*A*). In case of AP-1 complex, an antibody that recognized several members of the Fos family, including c-Fos, FosB, and Fos-related antigens (Fra-1 and Fra-2), caused a supershift, whereas c-Fos-specific antibody did not (Fig. 3*B*). Additional analysis demonstrated that Fra-1 member of the Fos family of transcription factors was a part of the AP-1 complex induced by glutamine starvation of TSE cells (Fig. 3*C*). The identity of the second subunit of the AP-1 complex was determined by inhibition of complex formation with the AP-1-binding site after

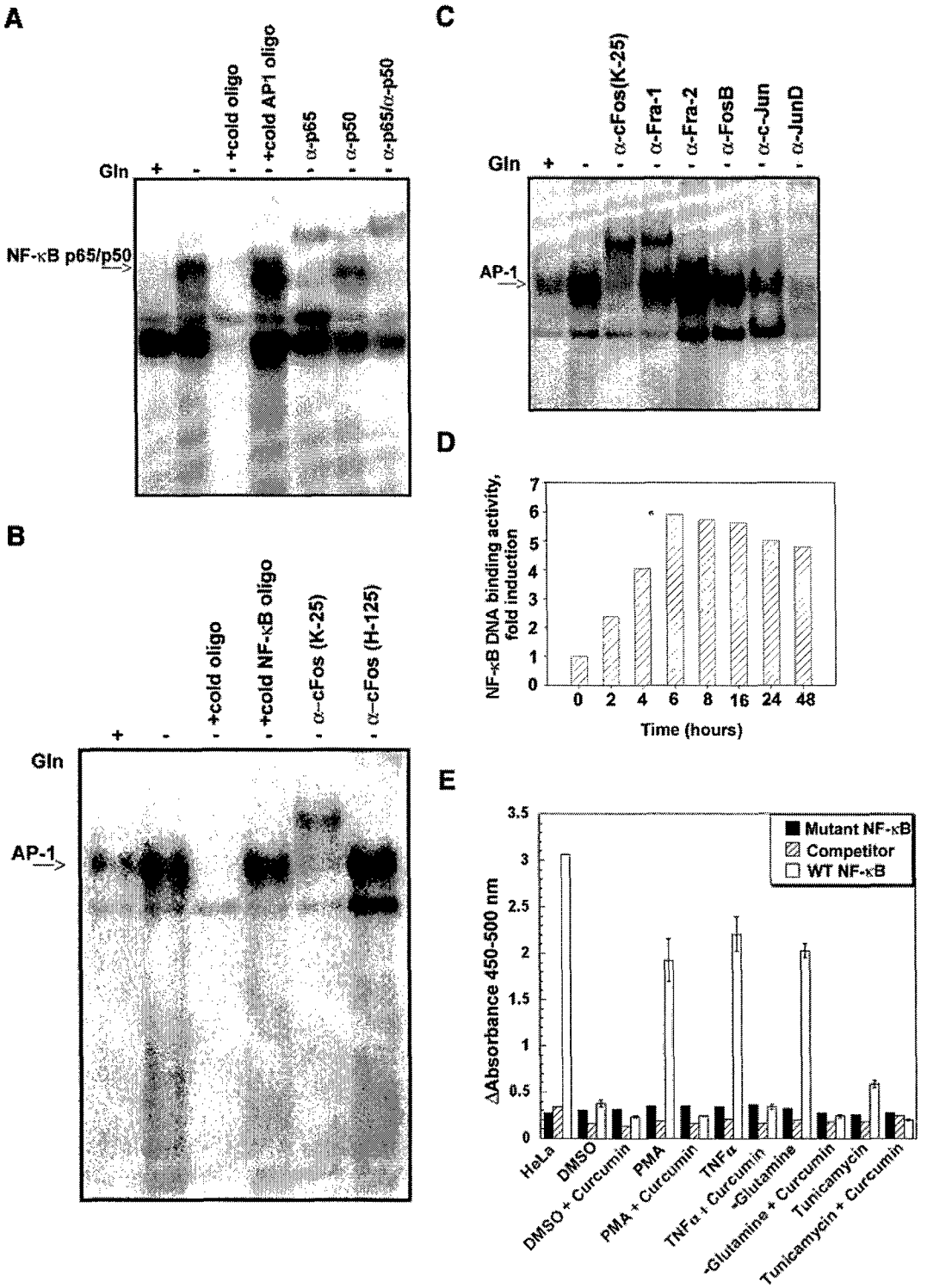


Fig. 3. Induction of NFκB and AP-1 DNA-binding activities by glutamine (*Gln*) starvation and inhibition by curcumin. Confluent TSE cells were glutamine starved for 8 h. Nuclear extracts were prepared and used in EMSAs with radiolabeled dsDNA containing NFκB-binding sites (*A*) and AP-1-binding sites (*B* and *C*). Binding mixtures were separated by electrophoresis, and bound radiolabeled dsDNAs were visualized by phosphorimagery. The position of the dsDNA complexed with NFκB p65/p50 and AP-1 is indicated. The composition of the NFκB and AP-1 DNA-bound complexes was determined using supershift analysis (see "Materials and Methods"). The specificity of the DNA binding was demonstrated by including 50-fold molar excess of cold dsDNA probe containing corresponding binding site in the binding mixture (+*cold oligo*) and by including 50-fold molar excess of cold unrelated dsDNA probe in the binding mixture. *D*, confluent TSE cells were glutamine starved for the times indicated. Nuclear extracts were prepared and used in a TransAM (Active Motif) ELISA-like assay to quantitate the NFκB p65 DNA-binding activity. Data were processed as described by the manufacturer, with correction for nonspecific binding of the transcription factor. Data represent the fold induction of DNA-binding activity relative to that in the 0-h, 4 mM glutamine sample. *E*, to test the effect of curcumin on NFκB activation, nuclear lysates were obtained from TSE cells treated with 0.1% DMSO carrier, 100 nM PMA, 20 ng/ml TNF-α, and 5 μg/ml tunicamycin or from glutamine-starved cells. Nuclear lysates were assayed for NFκB p65 DNA-binding activity using Mercury TransFactor (Clontech) ELISA-like kit. Data for wells with immobilized mutated κB element-containing DNA oligo, with immobilized wild-type (*WT*) κB element-containing DNA oligo, and with immobilized wild-type plus excess wild-type κB element-containing DNA oligo in solution (*Competitor*) are shown. Data represent the difference of the optical densities at the measurement (450 nm) and reference (500 nm) wavelengths after color development. Where *error bars* are shown, the data represent the means and SDs of triplicate wells. Nuclear extracts obtained from HeLa cells treated with 0.1 μg/ml TNF-α were used as a positive control.

addition of anti-c-Jun and anti-JunD-specific polyclonal antibody (Fig. 3*C*). The JunD antibodies caused considerably more inhibition of complex formation than the c-Jun antibodies, suggesting that JunD is the predominant AP-1-binding partner. Overexposure of these gels showed that these polyclonal antibodies did cause a faint supershift (data not shown).

TransAM ELISA-like assay was used to gain a quantitative assessment of NFκB p65 DNA-binding activity in nuclear extracts. The assay employs immobilized dsDNA corresponding to κB DNA elements to capture NFκB complexes and anti-RelA/p65 antibodies to detect bound transcription factors. Immobilized mutant DNA elements and excess dsDNA-containing wild-type elements are used to control for nonspecific factor binding. The time course of induction of NFκB p65 DNA-binding activity in response to glutamine starvation was examined (Fig. 3*D*). Within 2 h of glutamine starvation, NFκB p65 DNA-binding activity was induced approximately 2-fold and continued to increase within the next 4 h. NFκB p65 DNA-binding activity reached a maximum of 6-fold induction at 6 h, followed by a gradual decrease to 5-fold induction over the next 42 h. Thus, the time course of NFκB activation closely resembles but somewhat precedes the time courses of IL-8 and VEGF mRNA inductions in response to glutamine deprivation (see Fig. 1*C*). This is consistent with this transcription factor playing a causal role in the induction of IL-8 and VEGF transcription in response to glutamine deprivation.

We next tested the effect of pharmacological inhibition of NFκB and AP-1 activation by curcumin on the induction of VEGF and IL-8 expression. Curcumin (diferuloyl methane) is a yellow pigment component of the curry spice turmeric and has been used in traditional Indian medicine as an anti-inflammatory substance. Curcumin and derivatives are now being developed as cancer chemopreventative agents and as adjunct chemotherapeutic drugs. Curcumin is often used as a nontoxic pharmacological means to inhibit NFκB and AP-1 activation (48–51). The analysis was performed with cells treated with 5 μg/ml tunicamycin and with typical inducers of NFκB, tumor necrosis factor α (TNF-α), and phorbol 12-myristate 13-acetate (PMA) as well as glutamine-starved cells. Tunicamycin was selected as a specific inducer of the unfolded protein response. Mercury TransFactor assay was used to gain a quantitative assessment of the effectiveness of curcumin by measuring the effects on NFκB p65 DNA-binding activity in nuclear extracts. Nuclear extracts were prepared from TSE cells that were glutamine starved or treated with 0.1% (v/v) dimethyl sulfoxide (DMSO) as a carrier control, 100 nM PMA, 20 ng/ml TNF-α, or 5 μg/ml tunicamycin in the presence or absence of 50 μM curcumin (Fig. 3*E*). Nuclear extracts obtained from HeLa cells treated with 0.1 μg/ml TNF-α were used as a positive control. Glutamine starvation caused an approximately 5-fold induction of NFκB p65 DNA-binding activity. This level of NFκB DNA-binding activity induction was similar to that observed in TNF-α and PMA-treated TSE cells. In contrast, tunicamycin treatment resulted in only a 50% increase in NFκB p65 DNA-binding activity. The presence of curcumin completely abolished the up-regulation of NFκB DNA-binding activity by all of the inducers tested. Thus, glutamine deprivation caused an activation of NFκB that was comparable with those caused by PMA and TNF-α, and 50 μM curcumin was completely effective in blocking NFκB activation.

The effect of this dose of curcumin on VEGF and IL-8 mRNA expression was therefore examined. Triplicate cultures of confluent TSE cells were treated with either 0.1% (v/v) DMSO as a vehicle control or 5 μg/ml tunicamycin or were completely deprived of glutamine for 8 h in the presence or absence of 50 μM curcumin, and Northern blotting analyses were conducted (Fig. 4*A*). Curcumin was most effective at inhibiting the induction of IL-8 mRNA expression in response to glutamine starvation. Curcumin had no significant effect on VEGF and IL-8 mRNA levels in control, DMSO-treated cultures but did reduce the mRNA inductions in response to glutamine deprivation and tunicamycin treatment (Fig. 4*A*). In the absence of curcumin, glutamine deprivation and tunicamycin induced VEGF mRNA levels 6.5- and 6.3-fold, respectively. In the presence of 50 μM curcumin, glutamine deprivation and tunicamycin caused VEGF mRNA inductions of 3.7- and 4.4-fold, respectively. IL-8 mRNA expression was affected similarly by these agents. In the absence of curcumin, glutamine deprivation and tunicamycin treatment induced *IL-8* gene expression 14.3- and 6.5-fold, respectively. In the presence of 50 μM curcumin, IL-8 mRNA levels were induced 2.6-fold by glutamine starvation and 4.5-fold by tunicamycin. Thus, curcumin was able to inhibit the induction of VEGF and IL-8 mRNA expression in response to glutamine deprivation by 43 and 80%, respectively. The responses of VEGF and IL-8 expression to tunicamycin were both reduced by approximately 30%. The fact that curcumin completely inhibited NFκB activation and inhibited IL-8 mRNA induction by 80% is consistent with the response being primarily, but not completely, due to increased gene transcription that is dependent on NFκB and/or AP-1 activation.

To further test the roles of NFκB and AP-1 transcription factors in the control of VEGF and IL-8 transcription in response to glutamine deprivation, recombinant adenoviral vectors encoding the IκB super repressor (IκBM) and A-Fos were constructed and used. IκBM is a mutant form of IκBα with serine residues 32 and 36 mutated to alanine residues (52). This protein binds NFκB and sequesters it in the cytoplasm but is not phosphorylated and degraded in response to NFκB-activating conditions. Overexpression of IκBM therefore blocks NFκB nuclear translocation. The A-Fos dominant-negative mutant is an engineered c-Fos protein with an amphipathic acidic extension appended at the NH_2 terminus of the leucine zipper domain (43). The acidic extension heterodimerizes with the basic region of binding partners and thereby effectively blocks AP-1 DNA binding. Overexpression of A-Fos blocks formation of functional AP-1 complexes by sequestering c-FOS-binding partners in nonfunctional complexes. Confluent TSE cells were infected with recombinant adenoviruses in fetal bovine serum-free medium for 2 h. Infection with empty viral vector that does not contain a cDNA insert was performed to control for the effects of viral infection on gene expression. After the 2-h infection in fetal bovine serum-free medium, complete medium was added to the cells, and the virus was left on the cells for the next 22 h. After the 24-h infection, the cells expressing IκBM or A-Fos and the cells infected with the empty virus were treated with complete control medium or glutamine-free medium or were subjected to anoxia for 16 h. Total RNA was isolated, and Northern blotting analysis was performed (Fig. 4*B*). Expression of these dominant inhibitors demonstrated that NFκB and AP-1 transcription factors were necessary for the induction of IL-8 expression but dispensable for the induction of VEGF expression in response to glutamine starvation. IL-8 mRNA expression was induced by glutamine starvation in cells infected with the empty virus, and expression of IκBM and A-Fos inhibited the induction of IL-8 mRNA under glutamine-starved conditions by 87 and 82%, respectively. VEGF mRNA levels under glutamine-starved condition were only minimally affected by the expression of either dominant-negative mutant, with the IκBM and A-Fos causing 10 and 24.5% inhibition, respectively. Expression of A-Fos also decreased the induction of VEGF mRNA expression in response to anoxia by 29%. This result is in agreement with previous observations that AP-1 is involved in control of VEGF expression under hypoxic conditions (41, 53).

Analysis of NFκB DNA-binding activity in nuclear extracts from the IκBM and wild type IκBα (IκBwt) adenovirus-infected TSE cells revealed that overexpression of IκBM, as well as IκBwt, completely

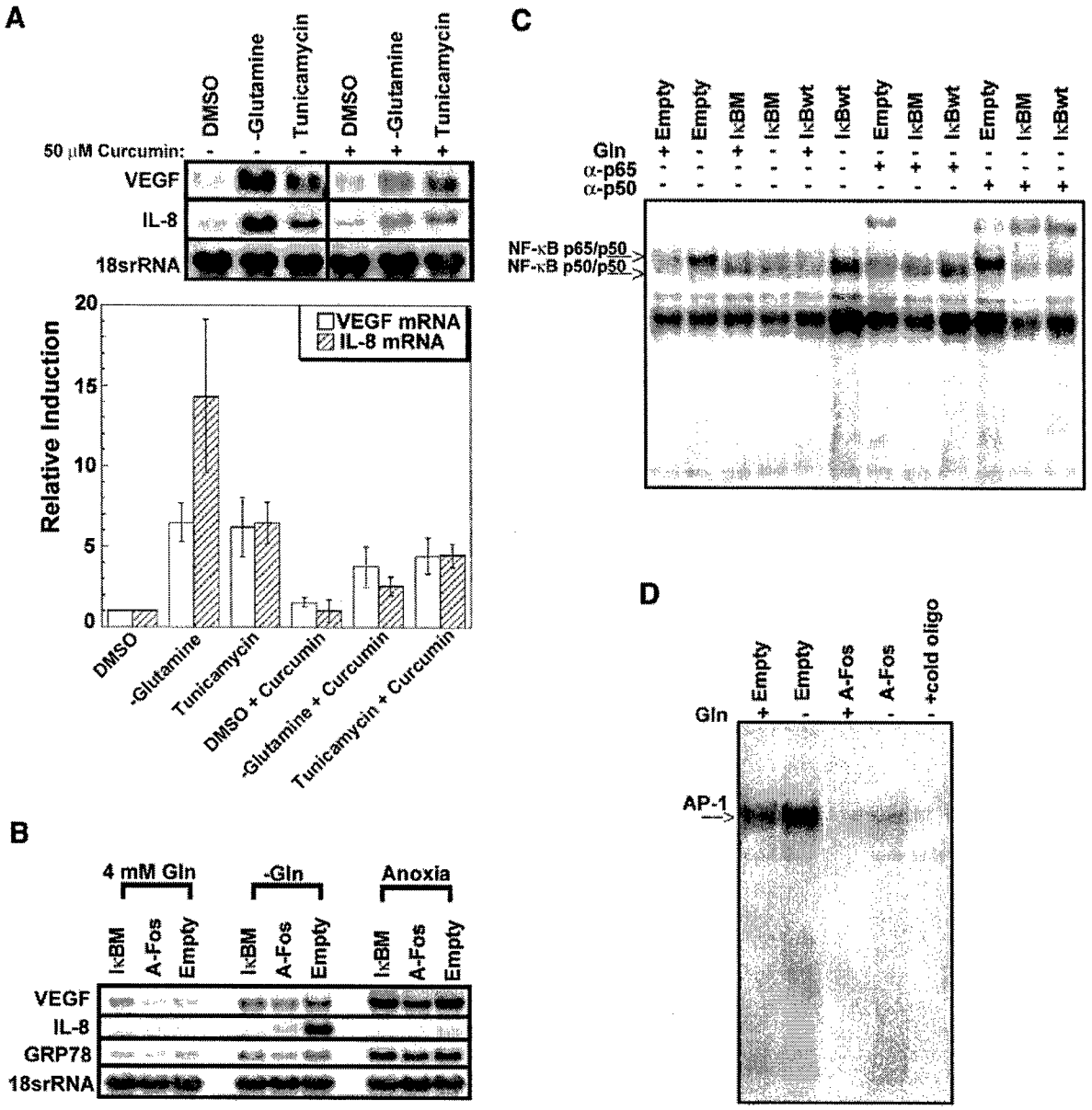
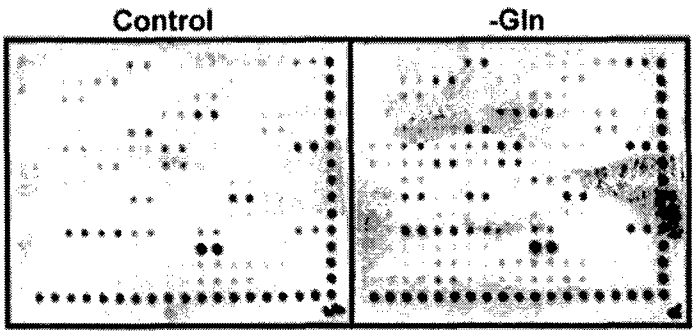


Fig. 4. Effects of pharmacological and genetic inhibition of NFκB and AP-1 on VEGF and IL-8 mRNA expression. *A*, to evaluate the effects of curcumin on VEGF and IL-8 mRNA expression, triplicate confluent cultures of TSE cells were grown in medium containing 0.1% DMSO, medium without glutamine (−*Glutamine*), or medium containing 5 μg/ml tunicamycin for 8 h in the presence or absence of 50 μM curcumin. Total RNA was isolated, and Northern blot analysis was performed. The data represent the mean mRNA levels ± SD. *B*, to evaluate the effect of genetically inhibiting NFκB and AP-1 activation, confluent TSE cells were infected with recombinant adenoviruses expressing IκBM or A-Fos or with an empty expression cassette as a control. Twenty-four h after infection, cells were glutamine (*Gln*) starved for 16 h. Anoxia treatment was used as a control for VEGF induction. Total RNA was isolated, and Northern blot analysis was performed. *C*, the effect of IκB overexpression on NFκB DNA-binding activity was evaluated. TSE cells were infected with recombinant adenoviruses overexpressing IκBM or wild type IκBα (IκBwt) or with an empty expression cassette. Twenty-four h after infection, cells were glutamine starved for 8 h. Nuclear extracts were isolated and used in EMSAs with radiolabeled dsDNA containing NFκB-binding site. The composition of the NFκB complex was determined employing supershift analysis with anti-NFκB p65 (*α-p65*) or anti-NFκB p50 (*α-p50*) antibodies. *D*, the effect of A-Fos overexpression on AP-1 DNA-binding activity was evaluated. TSE cells were infected with recombinant adenoviruses overexpressing A-Fos or with an empty expression cassette. Twenty-four h after infection, cells were glutamine starved for 8 h. Nuclear extracts were isolated and used in EMSAs with radiolabeled dsDNA containing AP-1-binding site.

excluded the NFκB p65 subunit from the DNA-binding complex, resulting in formation of inhibitory NFκB p50/p50 homodimers (Fig. 4*C*). The extent of exclusion was in keeping with the 87% reduction in IL-8 mRNA levels caused by overexpression of IκBM (Fig. 4*B*). However, it was surprising that IκB overexpression did not exclude all NFκB proteins from the nucleus but rather shifted the distribution of NFκB complexes in nuclear extracts. Adenovirus-mediated overexpression of A-Fos resulted in dramatically diminished AP-1 DNA-binding activity in nuclear extracts (Fig. 4*D*), in keeping with the observed 82% reduction in IL-8 mRNA levels due to A-Fos overexpression (Fig. 4*B*).

Effect of Ambient Glutamine Deprivation on Expression of NFκB Target Genes. To determine the specificity of IL-8 induction by glutamine deprivation and possibly identify NFκB-responsive genes besides *IL-8* that were induced during glutamine starvation of TSE cells, we performed a NFκB target gene array analysis (Fig. 5). RNA samples derived from control cells and cells starved for glutamine for 8 h (when IL-8 mRNA levels were at maximum; see Fig. 1*C*) were used to produce biotin-labeled cDNA probes that were hybridized to the array containing 111 NFκB target genes. This analysis confirmed the induction of IL-8 mRNA expression (spot positions H11/12; increased 8.8-fold) and demonstrated that only 17 of 111



	1/2	3/4	5/6	7/8	9/10	11/12	13/14	15/16	17/18
A	ALOX12	ADORA1	A1AT	A20	α1acidGP	AGT	APOC3	HLA-G1	ACTB
B	CD80	BCL-xl	BCL2A1	BGN	IFNβ	BDKRB1	BLR1	CCR5	
C	CD23	CD48	CD69	CD95	UGT8	c-myb	c-myc	MMP1	
D	F8	COX-2	c-rel	SCYB11	CYC-D1	CCND3	DDH1	ELAM-1	
E	SCYA11	F8	Fas-L	FTH	GAD65	Gal1-R	GAL-3	CSF3	
F	CSF2	GRO1	GSTP1	HMG-14	HMOX1	HAS1	ICAM-1	IFNγ	GAPDH
G	IGFBP1	IGFBP2	MAD-3	IL10	IL11	IL12	IL15	IL1-α	
H	IL1β	IL1RN	IL2	IL2-Ra	IL6	IL8	IL9	NOS	
I	IRF-1	IRF-2	JUN-B	LAMB2	Lox-1	LYZ	MAdCAM1	MCP-1	
J	CSF-1	MDR-1	MIP-2γ	MMP-3	MMP9	Mn-SOD	MTS1	NPYY1	
K	NFκB1	NFκB2	PRG1	p53	PAFR1	PAX6	PDGF-B	PTX3	UBC
L	TAP1	PENK	LMP-2	CD62	PTGIS	AGER	RANTES	SAA	
M	TCRB	TNC	THBS2	TNF	TNFβ	TNF-R	TGM1	UPAR	
N	VCAM-1	VEGFC	VIM	WT1	TNSFS5	MSX1	AhRR		

Fig. 5. Effect of glutamine (*Gln*) deprivation on NFκB target gene mRNA expression. The expression of 111 NFκB target genes in glutamine-starved and glutamine-fed TSE cells was analyzed using a commercial cDNA-cDNA hybridization-based gene array representing 111 NFκB target genes. Cell cultures were incubated in 4 mM glutamine-containing or glutamine-free media for 8 h, and then total RNA was isolated and used to produce biotin-labeled cDNA probes that were hybridized to the array membranes. The genes on the array are spotted in duplicate. The spots along the *right* and *bottom* sides of the array represent biotinylated DNA that has been spotted as a hybridization control. On each array, three housekeeping genes have been spotted on the *right* side of the array, from *top* to *bottom*: β-*actin* (*ACTB;* positions A17/18); *GAPDH* (positions F17/18); and *ubiquitin* (*UBC;* positions K17/18). Genes up-regulated by 2.0-fold or more include *IFN-β* (positions B9/10), *CYC-D1* (positions D9/10), *DDH1* (positions E13/14), *GRO1* (positions F3/4), *ICAM-1* (positions F13/14), *insulin-like growth factor binding protein 2* (*IGFBP2;* positions G3/4), *MAD-3* (positions G5/6), *IL15* (positions G13/14), *IL8* (positions H11/12), *IRF-2* (positions I3/4), *JUN-B* (positions I5/6), *NFκB2* (positions K3/4), *p53* (positions K7/8), *THBS2* (positions M3/4), *TNF-β* (positions M7/8), *vimentin* (*VIM;* positions N5/6), and *WT1* (positions N7/8).

NFκB target genes were up-regulated by at least 2.0-fold in response to glutamine deprivation. Genes up-regulated by 2.0-fold or more included: *IFN regulatory factor-2* (*IRF-2;* positions I3/4; 7.3-fold); *JUN-B* (positions I5/6; 6.1-fold); *dihydrodiol dehydrogenase-1* (*DDH1;* positions E13/14; 4.2-fold); *intercellular adhesion molecule 1* (*ICAM-1;* positions F13/14; 4.1-fold); Wilm's tumor suppressor gene (*WT1;* positions N7/8; 3.6-fold), *vimentin* (positions N5/6; 3.1-fold); *cyclin-D1* (*CYC-D1;* positions D9/10; 2.7-fold); *IκBα* (*MAD-3;* positions G5/6; 2.7-fold); *GROα/GRO1/CXCL1* (positions F3/4; 2.6-fold); *insulin-like growth factor binding protein 2* (*IGFBP2;* positions G3/4; 2.6-fold); *NFκB2* (*p100/p52;* positions K3/4; 2.2-fold); *p53 tumor suppressor* (*p53;* positions K7/8; 2.2-fold); *IFN-β* (positions B9/10; 2.2-fold); *IL-15* (positions G13/14; 2.0-fold); *thrombospondin-2* (*THBS2;* positions M3/4; 2.0-fold); and *TNF-β* (positions M7/8; 2.0-fold). The expression of three housekeeping genes spotted on the array were quantified: β-actin (*ACTB;* positions A17/18; 1.3-fold); *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH;* positions F17/18; 0.75-fold); and *ubiquitin* (*UBC;* positions K17/18; 1.7-fold). The extent of decreased GAPDH mRNA expression in glutamine-starved TSE cells is consistent with previous results (20, 46).

DISCUSSION

The present study demonstrated that a breast carcinoma cell line responded to glutamine deprivation by greatly increasing the expression of two pro-angiogenic factors, VEGF and IL-8. The response is partially due to mRNA stabilization. However, mRNA decay rates for these mRNAs were not decreased enough to totally account for the observed increases in steady-state mRNA levels. This is particularly true for IL-8 mRNA, for which mRNA level increased by as much as 2 orders of magnitude and mRNA half-life was increased by only 3-fold. The NFκB and AP-1 DNA-binding activities were induced by glutamine starvation. In the case of NFκB, the induction was pronounced and estimated to be 5–6-fold by a quantitative DNA-binding protein capture assay. AP-1 induction was less pronounced, for appreciable AP-1 DNA-binding activity was present in the nuclei of control, glutamine-fed cells. Furthermore, curcumin, which inhibits NFκB and AP-1 activation, inhibited the expression of VEGF and IL-8 mRNAs. The effect of curcumin was most dramatic for IL-8 mRNA expression, blocking its induction by 80%. Curcumin inhibited VEGF mRNA induction by only 40%. A specific genetic approach confirmed that NFκB and AP-1 transcription factors are mediators of the induction of *IL-8* gene expression but not *VEGF* gene expression. Similar to curcumin treatment, expression of IκBM and A-Fos inhibited the increase in IL-8 mRNA levels after glutamine deprivation by more than 80%. This level of inhibition is consistent with a mechanism of induction that is primarily, but not completely, transcriptional. IκBM (as well as IκBwt) and A-Fos expression were also quite effective in inhibiting the formation of NFκB p65/p50 complexes and AP-1 complexes, respectively. Surprisingly, expression of IκB proteins did not prevent p50/p50 complex formation in nuclear extracts. However, because p50/p50 complexes are inhibitory rather than transactivating, this is consistent with inhibition of IL-8 expression by blocking NFκB function.

Glutamine is the most abundant amino acid in the circulation and is metabolized by tumor tissues at much higher rates than any other amino acid. Glutaminolysis provides cells with metabolic precursors, energy, and reductive equivalents. Because glutamine is such an important nutrient, its absence from the cell culture medium may perturb cellular function and induce cellular stresses such as energy depletion, ER stress, oxidative stress, and osmotic stress. The present study shows for the first time that glutamine deprivation leads to the activation of transcription factors NFκB and AP-1. The particular stress and molecular mechanism that leads to these responses is not

yet known. Previous studies have demonstrated that supplemental glutamine feeding can reduce plasma IL-8 levels and the release of IL-8 by peripheral blood mononuclear cells in response to septic insults (54, 55). Recently, glutamine availability was shown to decrease the expression of IL-8 by IL-1β-treated monocytes and by LPS-treated intestinal cells (56, 57). However, there is only one published study that established NFκB activation in response to nutrient deprivation. While the present study was in progress, Jiang *et al.* (42) clearly demonstrated that leucine deprivation caused NFκB activation and that this response was dependent on phosphorylation of the eukaryotic initiation factor 2 α (eIF2α) by general control nonderepressible kinase 2 [GCN2 (EIF2AK4)]. IκBα serine phosphorylation and IκB degradation were not involved, but eIF2α activation did coincide with loss of IκBα binding to RelA/p65. A role for GCN2 is reminiscent of activation of NFκB by another eIF2α kinase, the double-stranded RNA-activated protein kinase PKR, in response to double-stranded RNA, IFN, and various cytokines (58). GCN2 is activated by amino acid deficiency through binding to uncharged transfer RNAs. Thus, activation of GCN2, and perhaps NFκB, would only occur when cells experienced amino acid limitations sufficient to suppress aminoacyl-tRNA synthetase reactions. Likewise, only one previously published study examined the role of glutamine in AP-1 activation. Rhoads *et al.* (59) found that feeding glutamine to previously glutamine-starved porcine IPEC-J2 cells and in rat IEC-6 cells caused a transient stimulation of AP-1 activity. The effect of glutamine deprivation on AP-1 activity was not examined by these authors, and the mechanism of AP-1 stimulation was not addressed. We found that AP-1 DNA-binding activity was increased by glutamine deprivation. Gel shift experiments suggested that the AP-1 complex in the nuclei of glutamine-starved cells contained Fra-1, c-Jun, and JunD proteins. We were unable to obtain a supershift with two different c-Fos antibodies that have been shown to function in EMSA (Fig. 3*B;* H-125 and data not shown). We have not compared the composition of the basal AP-1 complex present in glutamine-fed cells with that of glutamine-starved cells.

The induction of VEGF mRNA expression in response to glutamine deprivation was well correlated with the inductions of the ER stress-responsive genes *GRP78* and *GADD153*. Thus, we hypothesize that the expression of VEGF is responsive to an ER stress response pathway. VEGF (and IL-8) has been shown to be responsive to hypoxia. We have not proven that the hypoxia-inducible factors (HIF-1 and HIF-2) are not involved in the response of VEGF and IL-8 to glutamine deprivation. However, the levels of HIF-1α and HIF-2α proteins and hypoxia response element-binding activity were not appreciably increased by glutamine deprivation of TSE cells.[3] If an ER stress-response mechanism is responsible for the induction of VEGF by glutamine deprivation, it is probably not the unfolded protein response, because glutamine deprivation causes a minimal increase in GRP78 expression and GRP78 transcription is extremely responsive to the unfolded protein response. In addition, by monitoring the editing of X-box-binding protein mRNA, we have found that glutamine deprivation does not cause appreciable activation of the endonuclease activity of IRE1, a marker for activation of the unfolded protein response.[3] A recent study from our laboratory showed that induction of VEGF expression by ARPE-19 cells in response to homocysteine treatment was dependent on the ER stress-responsive transcription factor ATF4, which results from eIF2α kinase activation (60).

Several previous studies have implicated NFκB in control of VEGF transcription. Huang *et al.* (33, 34, 61) demonstrated that inhibition of NFκB activity by expression of IκBM suppressed the induction of IL-8 and VEGF in prostate, ovarian, and melanoma cancer cells. This inhibition also reduced tumorigenicity, angiogenesis, and metastasis of tumors formed from these cells. Bancroft *et al.* (26) published very similar results for a human head and neck squamous cell carcinoma cell line. However, a recent report from this same group showed that expression of IκBM blocked the induction of IL-8 but not VEGF in head and neck squamous cell carcinoma cells treated with epidermal growth factor (62). Likewise, in the present study, we observed that expression of IκBM effectively blocked the induction of IL-8 expression in response to glutamine deprivation without appreciably affecting the induction of VEGF expression. In addition, effective inhibition of NFκB activation by curcumin did not suppress VEGF mRNA expression nearly as much as IL-8 mRNA expression. We conclude that NFκB and AP-1 play key roles in the control of *IL-8* transcription and only minor roles in the control of *VEGF* transcription by glutamine in TSE cells.

In a recent publication, Lee *et al.* (63) presented an explanation for the often-observed coexpression of VEGF and IL-8. Using a cytokine gene array, these authors discovered that treatment of brain microvascular endothelial cells with VEGF caused severalfold increase in IL-8 expression. We have tested the possibility that IL-8 expression is secondary to autocrine stimulation of glutamine-starved cells by secreted VEGF. Treatment with conditioned media obtained from glutamine-starved cells, after repletion of glutamine, did not cause stimulation of IL-8 (or VEGF) expression.[4] In addition, glutamine starvation was able to increase IL-8 mRNA expression even when protein translation was inhibited by cycloheximide. Thus, we conclude that IL-8 expression in response to glutamine deprivation is not simply the result of VEGF accumulation in the culture media. Furthermore, in the same cytokine gene array experiment, Lee *et al.* (63) found that the expression of GROα was reduced severalfold by VEGF treatment. This is in sharp contrast to our finding that GROα was induced by glutamine deprivation.

Only a small subset of NFκB-inducible genes was responsive to glutamine deprivation. Interestingly, both IL-8 and GROα were responsive to glutamine deprivation. The up-regulation of GROα by glutamine deprivation and necessity of NFκB function for this response has been confirmed by Northern blotting analysis.[3] Like IL-8, GROα is a neutrophil chemoattractant that has been implicated in cancer progression. GROα is overexpressed in several tumors and associated with tumorigenicity, invasiveness, and metastatic ability (64–67). Additionally, both IL-8 and GROα stimulate the motility of breast cancer cell lines (68). Another mRNA up-regulated by glutamine deprivation was that of the intermediate filament vimentin. Vimentin expression, besides being a marker of epithelial to mesenchymal transition, is also associated with malignant progression, invasiveness, and metastasis in several tumors (for example, see Ref. 69).

In summary, we have shown that the expression of pro-angiogenic and pro-metastatic factors VEGF and IL-8 by TSE breast carcinoma cells is induced by glutamine deprivation. These inductions are partially due to mRNA stabilization. We also present the novel observation that glutamine deprivation activates NFκB and AP-1 transcription complexes. This is the first study to show AP-1 activation by nutrient deprivation and only the second study to show that nutrient deprivation of any kind causes NFκB activation. Furthermore, this study demonstrated that activation of NFκB and AP-1 transcription factors was necessary for the induction of IL-8 mRNA expression, but not VEGF expression, in response to glutamine starvation. Although the

[3] E. V. Bobrovnikova-Marjon, unpublished data.

[4] P. L. Marjon, unpublished data.

data indicate that VEGF transcription might be responsive to activation of ER stress response pathway(s) engaged during glutamine deprivation, the specific pathways and transcription factors involved remain unknown. The results suggest that low glutamine concentrations in the tumor microenvironment could contribute to angiogenic factor expression through numerous mechanisms. These mechanisms may represent novel targets for therapies directed at the angiogenic switch rather than specific angiogenic factors.

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REFERENCES

1. McCarty MF, Liu W, Fan F, et al. Promises and pitfalls of anti-angiogenic therapy in clinical trials. Trends Mol Med 2003;9:53–8.
2. Shima DT, Deutsch U, D'Amore PA. Hypoxic induction of vascular endothelial growth factor (VEGF) in human epithelial cells is mediated by increases in mRNA stability FEBS Lett 1995;370:203–8.
3. Ikeda E, Achen MG, Breier G, Risau W. Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. J Biol Chem 1995;270:19761–6.
4. Levy AP, Levy NS, Wegner S, Goldberg MA. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. J Biol Chem 1995;270:13333–40.
5. Xie K. Interleukin-8 and human cancer biology. Cytokine Growth Factor Rev 2001;12:375–91.
6. De Larco JE, Wuertz BR, Rosner KA, et al. A potential role for interleukin-8 in the metastatic phenotype of breast carcinoma cells. Am J Pathol 2001;158:639–46.
7. Miller LJ, Kurtzman SH, Wang Y, Anderson KH, Lindquist RR, Kreutzer DL. Expression of interleukin-8 receptors on tumor cells and vascular endothelial cells in human breast cancer tissue. Anticancer Res 1998;18:77–81.
8. Salcedo R, Martins-Green M, Gertz B, Oppenheim JJ, Murphy WJ. Combined administration of antibodies to human interleukin 8 and epidermal growth factor receptor results in increased antimetastatic effects on human breast carcinoma xenografts. Clin Cancer Res 2002;8:2655–65.
9. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. J Immunol 2003;170:3369–76.
10. Heidemann J, Ogawa H, Dwinell MB, et al. Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. J Biol Chem 2003;278:8508–15.
11. Benelli R, Morini M, Carrozzino F, et al. Neutrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. FASEB J 2002;16:267–9.
12. Koch AE, Polverini PJ, Kunkel SL, et al. Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science 1992;258:1798–801.
13. Reiland J, Furcht LT, McCarthy JB. CXC-chemokines stimulate invasion and chemotaxis in prostate carcinoma cells through the CXCR2 receptor. Prostate 1999;41:78–88.
14. Xie K. Interleukin-8 and human cancer biology. Cytokine Growth Factor Rev 2001;12:375–91.
15. Roslin M, Henriksson R, Bergstrom P, Ungerstedt U, Bergenheim AT. Baseline levels of glucose metabolites, glutamate and glycerol in malignant glioma assessed by stereotactic microdialysis. J Neurooncol 2003;61:151–60.
16. Kallinowskil F, Davel S, Vaupell P, Baessler KH, Wagner K. Glucose, lactate, and ketone body utilization by human mammary carcinomas in vivo. Adv Exp Med Biol 1985;191:763–73.
17. Stein I, Neeman M, Shweiki D, Itin A, Keshet E. Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes. Mol Cell Biol 1995;15:5363–8.
18. Shweiki D, Neeman M, Itin A, Keshet E. Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: implications for tumor angiogenesis. Proc Natl Acad Sci USA 1995;92:768–72.
19. Collins CL, Wasa M, Souba WW, Abcouwer SF. Regulation of glutamine synthetase in human breast carcinoma cells and experimental tumors. Surgery 1997;122:451–63.
20. Marjon PL, Bobrovnikova-Marjon EV, Abcouwer SF. Expression of the proangiogenic factors vascular endothelial growth factor and interleukin-8/CXCL8 by human breast carcinomas is responsive to nutrient deprivation and endoplasmic reticulum stress. Mol Cancer 2004;3:4.
21. Abcouwer SF, Marjon PL, Loper RK, Vander Jagt DL. Response of VEGF expression to amino acid deprivation and inducers of endoplasmic reticulum stress. Investig Ophthalmol Vis Sci 2002;43:2791–8.
22. Roebuck KA. Regulation of interleukin-8 gene expression. J Interferon Cytokine Res 1999;19:429–38.
23. Mastronarde JG, Monick MM, Mukaida N, Matsushima K, Hunninghake GW. Activator protein-1 is the preferred transcription factor for cooperative interaction with nuclear factor-κB in respiratory syncytial virus-induced interleukin-8 gene expression in airway epithelium. J Infect Dis 1998;177:1275–81.
24. Huang S, DeGuzman A, Bucana CD, Fidler IJ. Level of interleukin-8 expression by metastatic human melanoma cells directly correlates with constitutive NF-κB activity. Cytokines Cell Mol Ther 2000;6:9–17.
25. Takaya H, Andoh A, Shimada M, Hata K, Fujiyama Y, Bamba T. The expression of chemokine genes correlates with nuclear factor-κB activation in human pancreatic cancer cell lines. Pancreas 2000;21:32–40.
26. Bancroft CC, Chen Z, Dong G, et al. Coexpression of proangiogenic factors IL-8 and VEGF by human head and neck squamous cell carcinoma involves coactivation by MEK-MAPK and IKK-NF-κB signal pathways. Clin Cancer Res 2001;7:435–42.
27. Chua CC, Hamdy RC, Chua BH. Mechanism of transforming growth factor-β1-induced expression of vascular endothelial growth factor in murine osteoblastic MC3T3–E1 cells. Biochim Biophys Acta 2000;1497:69–76.
28. Yoshida S, Ono M, Shono T, et al. Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor α-dependent angiogenesis. Mol Cell Biol 1997;17:4015–23.
29. Eisma RJ, Spiro JD, Kreutzer DL. Role of angiogenic factors: coexpression of interleukin-8 and vascular endothelial growth factor in patients with head and neck squamous carcinoma. Laryngoscope 1999;109:687–93.
30. Fasciani A, D'Ambrogio G, Bocci G, Monti M, Genazzani AR, Artini PG. High concentrations of the vascular endothelial growth factor and interleukin-8 in ovarian endometriomata. Mol Hum Reprod 2000;6:50–4.
31. Bancroft CC, Chen Z, Dong G, et al. Coexpression of proangiogenic factors IL-8 and VEGF by human head and neck squamous cell carcinoma involves coactivation by MEK-MAPK and IKK-NF-κB signal pathways. Clin Cancer Res 2001;7:435–42.
32. Yuan A, Yu CJ, Luh KT, Kuo SH, Lee YC, Yang PC. Aberrant p53 expression correlates with expression of vascular endothelial growth factor mRNA and interleukin-8 mRNA and neoangiogenesis in non-small-cell lung cancer. J Clin Oncol 2002;20:900–10.
33. Huang S, Pettaway CA, Uehara H, Bucana CD, Fidler IJ. Blockade of NF-κB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. Oncogene 2001;20:4188–97.
34. Huang S, Robinson JB, Deguzman A, Bucana CD, Fidler IJ. Blockade of nuclear factor-κB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. Cancer Res 2000;60:5334–9.
35. Shi Q, Le X, Abbruzzese JL, et al. Cooperation between transcription factor AP-1 and NF-κB in the induction of interleukin-8 in human pancreatic adenocarcinoma cells by hypoxia. J Interferon Cytokine Res 1999;19:1363–71.
36. Collins TS, Lee LF, Ting JP. Paclitaxel up-regulates interleukin-8 synthesis in human lung carcinoma through an NF-κB- and AP-1-dependent mechanism. Cancer Immunol Immunother 2000;49:78–84.
37. Desbaillets I, Diserens AC, de Tribolet N, Hamou MF, Van Meir EG. Regulation of interleukin-8 expression by reduced oxygen pressure in human glioblastoma. Oncogene 1999;18:1447–56.
38. Damert A, Ikeda E, Risau W. Activator-protein-1 binding potentiates the hypoxiainducible factor-1-mediated hypoxia-induced transcriptional activation of vascular endothelial growth factor expression in C6 glioma cells. Biochem J 1997;327:419–23.
39. Xu L, Fukumura D, Jain RK. Acidic extracellular pH induces vascular endothelial growth factor (VEGF) in human glioblastoma cells via ERK1/2 MAPK signaling pathway: mechanism of low pH-induced VEGF. J Biol Chem 2002;277:11368–74.
40. Shih SC, Claffey KP. Role of AP-1 and HIF-1 transcription factors in TGF-β activation of VEGF expression. Growth Factors 2001;19:19–34.
41. Michiels C, Minet E, Michel G, Mottet D, Piret JP, Raes M. HIF-1 and AP-1 cooperate to increase gene expression in hypoxia: role of MAP kinases. IUBMB Life 2001;52:49–53.
42. Jiang HY, Wek SA, McGrath BC, et al. Phosphorylation of the α subunit of eukaryotic initiation factor 2 is required for activation of NF-κB in response to diverse cellular stresses. Mol Cell Biol 2003;23:5651–63.
43. Olive M, Krylov D, Echlin DR, Gardner K, Taparowsky E, Vinson C. A dominant negative to activation protein-1 (AP1) that abolishes DNA binding and inhibits oncogenesis. J Biol Chem 1997;272:18586–94.
44. Chomcynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
45. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA 1998;95:2509–14.
46. Abcouwer SF, Schwarz C, Meguid RA. Glutamine deprivation induces the expression of GADD45 and GADD153 primarily by mRNA stabilization. J Biol Chem 1999;274:28645–51.
47. Levy AP, Levy NS, Goldberg MA. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. J Biol Chem 1996;271:2746–53.
48. Park S, Lee DK, Yang CH. Inhibition of fos-jun-DNA complex formation by dihydroguaiaretic acid and in vitro cytotoxic effects on cancer cells. Cancer Lett 1998;127:23–8.
49. Jobin C, Bradham CA, Russo MP, et al. Curcumin blocks cytokine-mediated NF-κB activation and proinflammatory gene expression by inhibiting inhibitory factor I-κB kinase activity. J Immunol 1999;163:3474–83.
50. Xu YX, Pindolia KR, Janakiraman N, Chapman RA, Gautam SC. Curcumin inhibits IL1 α and TNF-α induction of AP-1 and NF-κB DNA binding activity in bone marrow stromal cells. Hematopathol Mol Hematol 1997;11:49–62.
51. Bierhaus A, Zhang Y, Quehenberger P, et al. The dietary pigment curcumin reduces endothelial tissue factor gene expression by inhibiting binding of AP-1 to the DNA and activation of NF-κ B. Thromb Haemostasis 1997;77:772–82.

52. Feig BW, Lu X, Hunt KK, et al. Inhibition of the transcription factor nuclear factor-κ B by adenoviral-mediated expression of I κ B α M results in tumor cell death. Surgery 1999;126:399–405.
53. Salnikow K, Kluz T, Costa M, et al. The regulation of hypoxic genes by calcium involves c-Jun/AP-1, which cooperates with hypoxia-inducible factor 1 in response to hypoxia. Mol Cell Biol 2002;22:1734–41.
54. de Beaux AC, O'Riordain MG, Ross JA, Jodozi L, Carter DC, Fearon KC. Glutamine-supplemented total parenteral nutrition reduces blood mononuclear cell interleukin-8 release in severe acute pancreatitis. Nutrition 1998;14:261–5.
55. Lin MT, Saito H, Furukawa S, et al. Alanyl-glutamine enriched total parenteral nutrition improves local, systemic, and remote organ responses to intraperitoneal bacterial challenge. J Parenter Enteral Nutr 2001;25:346–51.
56. Huang Y, Li N, Liboni K, Neu J. Glutamine decreases lipopolysaccharide-induced IL-8 production in Caco-2 cells through a non-NF-κB p50 mechanism. Cytokine 2003;22:77–83.
57. Coeffier M, Marion R, Ducrotte P, Dechelotte P. Modulating effect of glutamine on IL-1β-induced cytokine production by human gut. Clin Nutr 2003;22:407–13.
58. Williams BR. PKR: a sentinel kinase for cellular stress. Oncogene 1999;18: 6112–20.
59. Rhoads JM, Argenzio RA, Chen W, et al. L-Glutamine stimulates intestinal cell proliferation and activates mitogen-activated protein kinases. Am J Physiol 1997;272: G943–53.
60. Roybal CN, Yang S, Sun CW, et al. Homocysteine increases the expression of VEGF by a mechanism involving endoplasmic reticulum stress and transcription factor ATF4. J Biol Chem 2004;279:14844–52.
61. Huang S, DeGuzman A, Bucana CD, Fidler IJ. Nuclear factor-κB activity correlates with growth, angiogenesis, and metastasis of human melanoma cells in nude mice. Clin Cancer Res 2000;6:2573–81.
62. Bancroft CC, Chen Z, Yeh J, et al. Effects of pharmacologic antagonists of epidermal growth factor receptor, PI3K and MEK signal kinases on NF-κB and AP-1 activation and IL-8 and VEGF expression in human head and neck squamous cell carcinoma lines. Int J Cancer 2002;99:538–48.
63. Lee TH, Avraham H, Lee SH, Avraham S. Vascular endothelial growth factor modulates neutrophil transendothelial migration via up-regulation of interleukin-8 in human brain microvascular endothelial cells. J Biol Chem 2002;277:10445–51.
64. Loukinova E, Chen Z, Van Waes C, Dong G. Expression of proangiogenic chemokine Gro 1 in low and high metastatic variants of Pam murine squamous cell carcinoma is differentially regulated by IL-1α, EGF and TGF-β1 through NF-κB dependent and independent mechanisms. Int J Cancer 2001;94:637–44.
65. Lane BR, Liu J, Bock PJ, et al. Interleukin-8 and growth-regulated oncogene α mediate angiogenesis in Kaposi's sarcoma. J Virol 2002;76:11570–83.
66. Loukinova E, Dong G, Enamorado-Ayalya I, et al. Growth regulated oncogene-α expression by murine squamous cell carcinoma promotes tumor growth, metastasis, leukocyte infiltration and angiogenesis by a host CXC receptor-2 dependent mechanism. Oncogene 2000;19:3477–86.
67. Moore BB, Arenberg DA, Stoy K, et al. Distinct CXC chemokines mediate tumorigenicity of prostate cancer cells. Am J Pathol 1999;154:1503–12.
68. Youngs SJ, Ali SA, Taub DD, Rees RC. Chemokines induce migrational responses in human breast carcinoma cell lines. Int J Cancer 1997;71:257–66.
69. Fuchs IB, Lichtenegger W, Buehler H, et al. The prognostic significance of epithelial-mesenchymal transition in breast cancer. Anticancer Res 2002;22:3415–9.

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Expression of the pro-angiogenic factors vascular endothelial growth factor and interleukin-8/CXCL8 by human breast carcinomas is responsive to nutrient deprivation and endoplasmic reticulum stress

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Abstract

Background: The expression of pro-angiogenic cytokines, such as vascular endothelial growth factor (VEGF) and interleukin-8/CXCL8 (IL-8), plays an important role in tumor growth and metastasis. Low oxygen tension within poorly-vascularized tumors is thought to be the prime stimulus causing the secretion of VEGF. The expression of IL-8 by solid tumors is thought to be primarily due to intrinsic influences, such as constitutive activation of nuclear factor kappa B (NF-κB). However, VEGF expression is responsive to glucose deprivation, suggesting that low concentrations of nutrients other than oxygen may play a role in triggering the pro-angiogenic phenotype. Glucose deprivation causes endoplasmic reticulum (ER) stress and alters gene expression through the unfolded protein response (UPR) signaling pathway. A branch of the UPR, known as the ER overload response (EOR), can cause NF-κB activation. Thus, we hypothesized that treatments that cause ER stress and deprivation of other nutrients, such as amino acids, would trigger the expression of angiogenic cytokines by breast cancer cell lines.

Results: We found that glutamine deprivation and treatment with a chemical inducer of ER stress (tunicamycin) caused a marked induction of the secretion of both VEGF and IL-8 protein by a human breast adenocarcinoma cell line (TSE cells). Glutamine deprivation, glucose deprivation and several chemical inducers of ER stress increased VEGF and IL-8 mRNA expression in TSE and other breast cancer cell lines cultured under both normoxic and hypoxic conditions, though hypoxia generally diminished the effects of glucose deprivation. Of all amino acids tested, ambient glutamine availability had the largest effect on VEGF and IL-8 mRNA expression. The induction of VEGF mRNA expression, but not IL-8, was sustained and closely corresponded with the upregulated expression of the ER stress-responsive genes glucose-regulated protein 78 (GRP78) and growth arrest and DNA damage inducible gene 153 (GADD153).

Conclusion: These results suggest that nutrient deprivation within the solid tumor microenvironment might contribute to the activation of a pro-angiogenic phenotype. The angiogenic switch may act to increase blood supply in response to nutrient deprivation as well as hypoxia.

Background

It has become apparent that some aspects of malignant progression are mediated through the effects of the tumor microenvironment. Environmental conditions affect the regulation of gene expression by both cancer cells and stromal cells within the tumor. As tumors grow in size, they must respond and adapt to a nutrient-limited environment. Neoplastic progression includes genetic alterations that allow malignant cells to ignore normal growth controls. For these cells, growth is limited by the delivery rate of oxygen and nutrients (such as glucose and amino acids) and removal rate of waste products (such as CO_2 and lactic acid) [1]. As a tumor grows in size, the cancer cells and the stromal cells that surround them both experience progressive hypoxia, nutrient starvation and acidosis until the tumor microenvironment becomes deleterious to growth or even toxic [1]. These cells survive and adapt to this ischemic environment by producing pro-angiogenic factors to initiate the formation and attraction of new blood vessels to the tumor [2]. In fact, an increase in the synthesis of pro-angiogenic factors is a natural cellular response to an ischemic environment [3].

Many cancer researchers seem to associate ischemia only with a lack of oxygen delivery. For this reason, much attention has been devoted to understanding the way that tumor cells respond and adapt to oxygen limitation. Tumor hypoxia is a very active research area. We now know that many genes important to both tumor cell energy metabolism and angiogenesis are responsive to hypoxia [4]. By far the most studied hypoxia-responsive gene is vascular endothelial growth factor A, the original member of the vascular permeability factor (VPF)/VEGF family of proteins. Vascular endothelial growth factor A (hereafter referred to as VEGF) is recognized as the single most important angiogenic factor [5]. VEGF is a multifunctional cytokine that is widely expressed by tumor cells and increases microvascular permeability, induces endothelial cell migration and division, and promotes endothelial cell survival [6]. It may also act as an autocrine survival factor for some cancer cells [7,8]. Plasma levels of VEGF have prognostic value for several cancers, including breast cancer [9,10].

In a tumor which has outgrown the existing vasculature, ischemia results in hypoxia that induces the transcription of VEGF through a well-defined signaling mechanism [4]. This response to hypoxia stimulates angiogenesis to augment the existing vasculature and enable the delivery of greater amounts of oxygen, effectively alleviating the hypoxic stress. However, the hypoxic response mechanism does not always completely explain the increased VEGF expression by ischemic tissues. There are several studies suggesting that hypoxia is not the cause of heightened VEGF expression by ischemic tissues or tumors [11-16].

Several previous studies demonstrated that glucose deprivation also induces the expression of VEGF in cultured cell lines, including HepG2 [17] and Hep3B [11] human hepatomas, U-937 human monocytic cell line [18], C6 rat glial tumor cell line [19], and ARPE-19 human retinal pigmented epithelial cell line [20]. However, none of these studies demonstrated a mechanism for this effect. Glucose deprivation and treatments that cause ER dysfunction lead to the stressful accumulation of malfolded proteins within the ER, resulting in an endoplasmic reticulum stress response (ERSR) and activation of a signal transduction pathway known as the unfolded protein response (UPR). The transcription of several ER stress-responsive genes is highly induced by activation of the UPR, including glucose-regulated protein 78 (GRP78) which encodes the immunoglobin binding protein (BiP), and the growth arrest and DNA damage inducible gene 153 (GADD153) which encodes the CEBP-homologous protein (CHOP10). Because there are several studies demonstrating the activation of ERSR pathways and the expression of ER stress-responsive genes in tumor tissue [21-26], we reasoned that similar mechanisms could operate in cancer cells and, if so, then this could contribute to the expression of VEGF by poorly vascularized tumors.

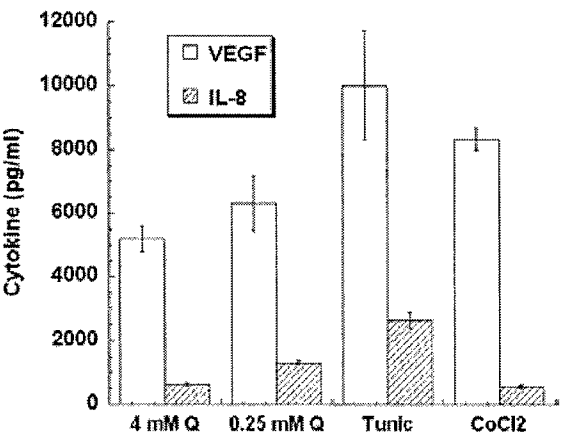

Figure 1
Secretion of VEGF and IL-8 protein by TSE cells treated with low glutamine-containing media, tunicamycin, and cobalt chloride. Triplicate cultures of confluent TSE cells in six-well plates were incubated with 3 mL per well of complete medium containing 4 mM glutamine (Q), 0.25 mM glutamine, 5 μg/mL tunicamycin (Tunic) or 100 μM $CoCl_2$ for 24 hours. A commercial ELISA was used to determine the amount of VEGF and IL-8 protein in the media. Data represent the mean concentration ± SD.

In addition, we sought to determine if expression of the CXC cytokine interleukin-8/CXCL8 (IL-8) was also responsive to nutrient deprivation and ER stress. IL-8 was first characterized as a neutrophil chemoattractant [27]. Soon after its discovery it was identified as a pro-angiogenic factor, but its role in cancer has only recently been fully appreciated [28]. Aberrant IL-8 expression has been documented in several solid tumor types, including breast [28]. IL-8 seems to function as both a pro-angiogenic and a pro-metastatic factor [29-35]. Like VEGF, high serum levels of IL-8 have been shown to be a negative prognostic indicator in several cancers [36-39]. IL-8 expression is highly dependent upon activity of nuclear factor kappa B (NF-κB) [40-42]. In turn, NF-κB can be activated by ER stress, through a UPR signaling pathway that has been described as the ER overload response (EOR) [43]. However, to the best of our knowledge, the effect of nutrient deprivation and ER stress on IL-8 expression has not yet been described.

In the present study, a human breast carcinoma cell line (TSE cells) was used as the model system to test the hypothesis that VEGF and IL-8 expression by tumor cells is induced by nutrient deprivation and ER stress. The expression of VEGF and IL-8 was compared to that of ER and nutrient stress-responsive genes GRP78 and GADD153, as well as the hypoxia-responsive gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results show that VEGF expression by TSE cells, as well as other breast carcinoma cell lines, is highly responsive to nutrient deprivation and treatments that cause ER stress. In addition, we present the novel observation that the expression of IL-8 is responsive to nutrient deprivation and ER stress. These findings suggest that a low concentration of nutrients other than oxygen in the tumor microenvironment, or any stress that disrupts ER function, has the potential to increase tumor angiogenesis and metastasis.

Results

Effect of glutamine deprivation, tunicamycin, and cobalt chloride on the secretion of VEGF and IL-8 protein by TSE cells

To determine if VEGF and IL-8 proteins were secreted by TSE cells under stress, as induced by glutamine deprivation, tunicamycin (an inhibitor of protein glycosylation in the ER), or hypoxia (mimicked by $CoCl_2$ [44]), triplicate confluent cultures of the various cell types were grown in 6-well plates and incubated with 3 mL per well of complete media containing 4 mM glutamine as a control, 0.25 mM glutamine, 5 μg/mL tunicamycin, or 100 μM $CoCl_2$ for 24 hours. ELISA was used to determine accumulated VEGF and IL-8 protein contents in the media (Figure 1). At 4 mM glutamine (the control condition), TSE cells showed a basal secretion of 5200 pg/mL VEGF protein. VEGF secretion increased to 6310 pg/mL with low glutamine conditions, to 10020 pg/mL with tunicamycin treatment and to 8340 pg/mL with $CoCl_2$ treatment. Secreted IL-8 protein concentrations were 610 pg/mL with control conditions, 1310 pg/mL with low glutamine, 2640 pg/mL with tunicamycin treatment, and 560 pg/mL with $CoCl_2$ treatment. Thus, TSE cells demonstrated an increased expression and secretion of both VEGF and IL-8 protein during conditions of glutamine deprivation and ER stress.

Response of VEGF and IL-8 mRNA expression to ER stress and nutrient deprivation under normoxic (20% O_2) and hypoxic (2% O_2) culture conditions

To test the hypothesis that VEGF is an ER stress-responsive gene with characteristics of expression similar to other ER stress-responsive genes, and that the mechanism of VEGF mRNA upregulation through ER stress is distinct from the well characterized hypoxia response of VEGF, TSE cells were simultaneously subjected to ER stress or nutrient deprivation under normoxic and hypoxic conditions. TSE cells were grown to confluence, rinsed twice with PBS and fed with either complete control medium, glutamine-free medium, glucose-free medium, medium containing 5 μg/mL tunicamycin, medium containing 10 μM A23187 (a calcium ionophore which releases ER calcium stores), medium containing 10 μg/mL Brefeldin A (an inhibitor of ER-to-Golgi vesicle transport) or medium containing 0.1% (v/v) DMSO as a vehicle control. Cells under the above conditions were treated in duplicate and cultured either under normoxic (20% O_2) or hypoxic (2% O_2) conditions for 24h. Total RNA was isolated and Northern blotting analyses was performed (Figure 2A). Results were compared to the untreated normoxic control.

This analysis showed that under normoxic conditions nutrient deprivation and chemical inducers of ER stress increase VEGF, GRP78 and GADD153 mRNA levels in similar fashions. VEGF mRNA expression was increased to the greatest extent (6-fold) in response to glutamine deprivation. VEGF mRNA expression was induced 4-fold in response to glucose deprivation, 3-fold in response to tunicamycin treatment, 5-fold in response to A23187 and 4-fold in response to Brefeldin A. GRP78 and GADD153 mRNA expression increased the most following Brefeldin A treatment (11-fold and 14-fold, respectively). After 24 h of treatment, IL-8 mRNA expression was increased only in response to glutamine deprivation, glucose deprivation and treatment with A23187 (2-fold, 3-fold and 22-fold, respectively). Under hypoxic conditions, VEGF mRNA was increased 4-fold in the complete media control sample compared to the VEGF mRNA level in the normoxic control sample, confirming VEGF's well-characterized response to hypoxia. Under hypoxic conditions, glutamine deprivation, tunicamycin, A23187, and Brefeldin A all caused further inductions of VEGF mRNA levels

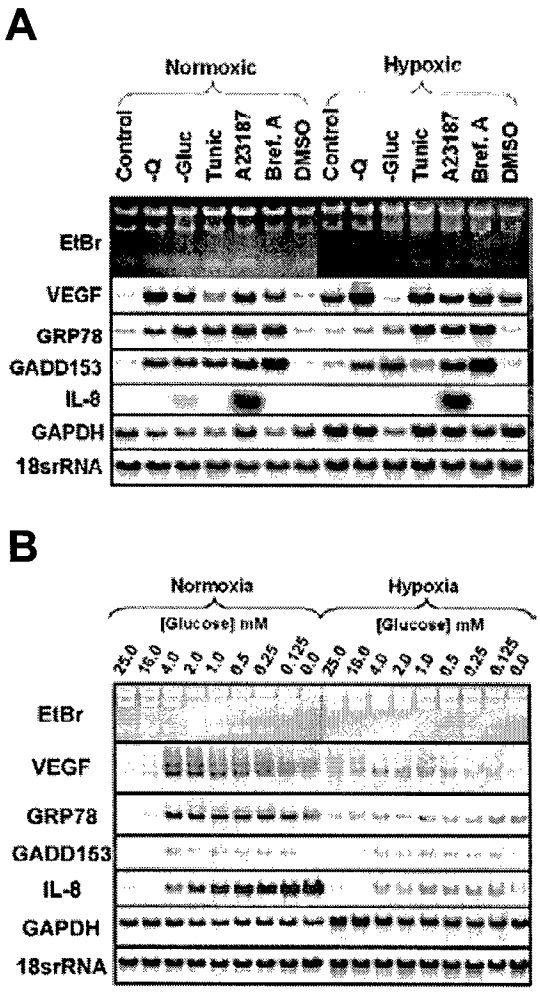

Figure 2
VEGF, IL-8, GRP78, GADD153 and GAPDH mRNA expression by TSE cells following glutamine deprivation, glucose deprivation and treatment with chemical inducers of ER stress under normoxic or hypoxic conditions. (A) Confluent TSE cells were fed with complete control medium, glutamine-free medium (-Q), glucose-free medium (-Gluc), medium containing 5 μg/mL tunicamycin (Tunic), medium containing 10 μM A23187, medium containing 10 μg/mL Brefeldin A (Bref. A), or medium containing 0.1% (v/v) DMSO as a vehicle control and cultured under either normoxic (20% O_2) or hypoxic (2% O_2) conditions. Total RNA was isolated and Northern blotting analysis performed. (B) Confluent TSE cells were cultured in media containing the indicated concentrations of glucose while under normoxic (20% O_2) or hypoxic (2% O_2) conditions for 24 hours. Total RNA was isolated and Northern blotting analysis performed.

compared to the hypoxic control. Glutamine deprivation under hypoxic conditions raised VEGF mRNA levels 12-fold, twice the induction observed when TSE cells were glutamine starved under normoxic conditions. GAPDH mRNA was raised slightly under all hypoxic conditions. Of notice was the fact that glucose deprivation under normoxic conditions induced VEGF and GRP78 mRNAs 4-fold and 8-fold, respectively, but glucose deprivation under hypoxic conditions failed to induce expression of VEGF mRNA at all, and the induction of GRP78 mRNA fell to 3-fold.

To confirm the observation that hypoxia impairs the induction of VEGF and GRP78 mRNA normally seen as a result of glucose deprivation, TSE cells were grown to confluence rinsed twice with PBS and fed with DMEM containing various concentrations of glucose: 0.0, 0.125, 0.25, 0.5, 1, 2, 4, 16 or 25 mM. Dialyzed FBS was used to supplement the media to prevent serum-derived glucose from being added. Duplicate samples were then cultured in hypoxic (2% O_2) or normoxic (20% O_2) conditions for 24 hours. Total RNA was isolated and Northern blotting analysis for VEGF, GRP78, GADD153, IL-8 and GAPDH mRNA was performed (Figure 2B). Samples were compared with the normoxic control (25 mM glucose). Under normoxic conditions, VEGF and GRP78 mRNAs were maximally induced at 2 mM glucose to 7-fold and 13-fold, respectively. In contrast, under hypoxic conditions inductions of VEGF and GRP78 mRNAs were almost completely suppressed. Hypoxia suppressed GADD153 mRNA inductions in response to glucose deprivation as well, by about a multiple of 2 at all concentrations below 2 mM glucose. Under normoxic conditions, GADD153 mRNA was maximally induced 17-fold at 1 mM glucose. However, at the same concentration of glucose under hypoxic conditions GADD153 mRNA was induced only 8-fold. Under normoxic conditions, IL-8 mRNA was greatly induced in a dose-dependent manner in response to glucose deprivation, being maximally induced 43-fold at 0.125 mM glucose and similarly induced as glucose concentrations were reduced to zero. Thus, this dose-response experiment confirmed that IL-8 mRNA expression was induced by glucose deprivation. However, inexplicably, a much greater level of induction of IL-8 expression by glucose deprivation was obtained in this experiment than in the previous experiment (Figure 2A). The induction of IL-8 was greatly reduced under hypoxic conditions. For example, at 0.125 mM glucose IL-8 mRNA was only induced by about 4-fold in the presence of hypoxia. Thus, the effect of glucose deprivation upon the expression of VEGF, IL-8 and other ER stress-responsive genes was retained but markedly reduced under hypoxic conditions.

Expression of VEGF and ER stress-responsive genes was also examined and compared in TSE, MDA-MB-231, MDA-MB-453, T47D, and MCF-7 human breast cancer cell lines subjected to nutrient deprivation and ER stress for 8 h under normoxic and hypoxic conditions. Confluent cells were fed with complete control medium, glutamine-free medium, glucose-free medium, medium containing 5 μg/mL tunicamycin, 500 nM thapsigargin, 10 μM A23187, 1 mM DTT, 10 μg/mL Brefeldin A, or medium containing 0.1% (v/v) DMSO as a vehicle control. The cultures were then placed in 20% O_2 or 2% O_2-containing environments for 8 hours. Total RNA was isolated and Northern blot analysis performed (Supplemental Figure 1 [see Additional File 1]). In response to glutamine deprivation, VEGF mRNA expression was similarly increased in all cell lines, with the greatest increase in MDA-MB-231 (2.3-fold). Glucose deprivation increased VEGF mRNA expression in every cell line tested with the greatest induction seen in T47D cells (2.9-fold). Tunicamycin, thapsigargin, Brefeldin A and A23187 increased VEGF mRNA expression in all cell lines tested. The MDA-MB-231 cell line increased the expression of VEGF mRNA to the greatest extent following treatment with the chemical inducers of ER stress, tunicamycin (2.5-fold), thapsigargin (2.5-fold), A23187 (5.0-fold) and Brefeldin A (3.2-fold). Under normoxic conditions, treatment with DTT induced the expression of VEGF mRNA appreciably only in the MDA-MB-231 cell line (1.9-fold).

Hypoxia alone increased VEGF mRNA expression in all cell lines tested, with MDA-MB-453 cell line demonstrating the greatest induction (3.3-fold). Upon glutamine deprivation under hypoxic conditions, TSE (3.3-fold), T47D (3.6-fold) and MCF-7 (2.9-fold) cell lines showed additionally increased VEGF mRNA expression compared to the hypoxic control. Under hypoxic conditions, 8 h of glucose deprivation also additionally upregulated VEGF mRNA expression compared to the hypoxic control lanes in TSE (2.9-fold) and MDA-MB-231 (8.8-fold). Glucose deprivation under hypoxic conditions repressed the expression of VEGF mRNA compared to the hypoxic control lanes in the MCF-7, T47D and MDA-MB-453 cancer cell lines. Most treatments with chemical inducers of ER stress during hypoxic culture conditions resulted in VEGF mRNA levels that were higher than those produced by hypoxia alone in the MCF-7, MDA-MB-231 and MDA-MB-453 cell line. In T47D cells, only A23187 treatment resulted in greater (4.0-fold) VEGF mRNA levels than hypoxia alone.

Expression of IL-8 mRNA was detected only in TSE and MDA-MB-231 cells, and was markedly increased under both normoxia and hypoxia by treatment with glutamine-free medium, glucose-free medium, thapsigargin, A23187 and Brefeldin A. The magnitude of inductions was not quantifiable because IL-8 mRNA was essentially undetected in control samples. However, A23187 induced the greatest IL-8 responses, tunicamycin caused relatively slight inductions and DTT did not appreciably induce IL-8 expression in either TSE or MDA-MB-231 cells. Surprisingly, hypoxia did not appreciably induce IL-8 mRNA expression in any of these breast cell lines. Both tunicamycin and DTT caused dramatic inductions of GRP78 mRNA expression in each cell. GADD153 expression by these cells was appreciably responsive to tunicamycin, but not to DTT treatment.

Response of VEGF, GRP78, GADD153 and IL-8 mRNA expression to amino acid deprivation

To determine if VEGF mRNA expression is responsive to the deprivation of amino acids other than glutamine, TSE cells were incubated in media lacking one or all of the amino acids found in modified Eagle's medium (MEM). TSE cells adapted to MEM were grown to confluence, rinsed twice and re-fed complete MEM, MEM lacking all amino acids, or MEM deficient in one of each of the 13 amino acids present in complete MEM and incubated for 12 hours. Dialyzed FBS was used to negate the contribution of amino acids within the sera. Total RNA was isolated and Northern blotting analyses were performed (Figure 3B). The amount of VEGF, GRP78, GADD153, IL-8 and GAPDH mRNA was determined relative to the amount of 18S rRNA within each sample and mRNA contents were compared to that of control cells in complete MEM and amino acid-free MEM (Figure 3A). VEGF mRNA expression was most responsive to glutamine deprivation (9-fold). Deprivation of other amino acids elicited smaller inductions. For example, lack of arginine induced VEGF mRNA 7.6-fold and lack of leucine, lysine and methionine each induced VEGF mRNA approximately 5-fold. Deprivation of all 13 amino acids induced VEGF mRNA 5.6-fold. GRP78 mRNA was induced to a lesser extent than VEGF by deprivation of various amino acids. Glutamine deprivation had the greatest effect on GRP78 mRNA inducing it 2.3-fold. GADD153 mRNA was also more responsive (3.9-fold) to glutamine deprivation than to a lack of other amino acids. Total amino acid starvation resulted in a marked 12.3-fold induction of GADD153. At this time point, IL-8 expression was only responsive to glutamine deprivation, with an induction of slightly more than 2-fold. However, IL-8 mRNA expression was greatly induced 12-fold by total amino acid starvation. Comparison of VEGF with GADD153 mRNA levels yielded a good correlation ($r = 0.79$). Comparison of VEGF with GRP78 mRNA levels yielded a decreased correlation ($r = 0.63$).

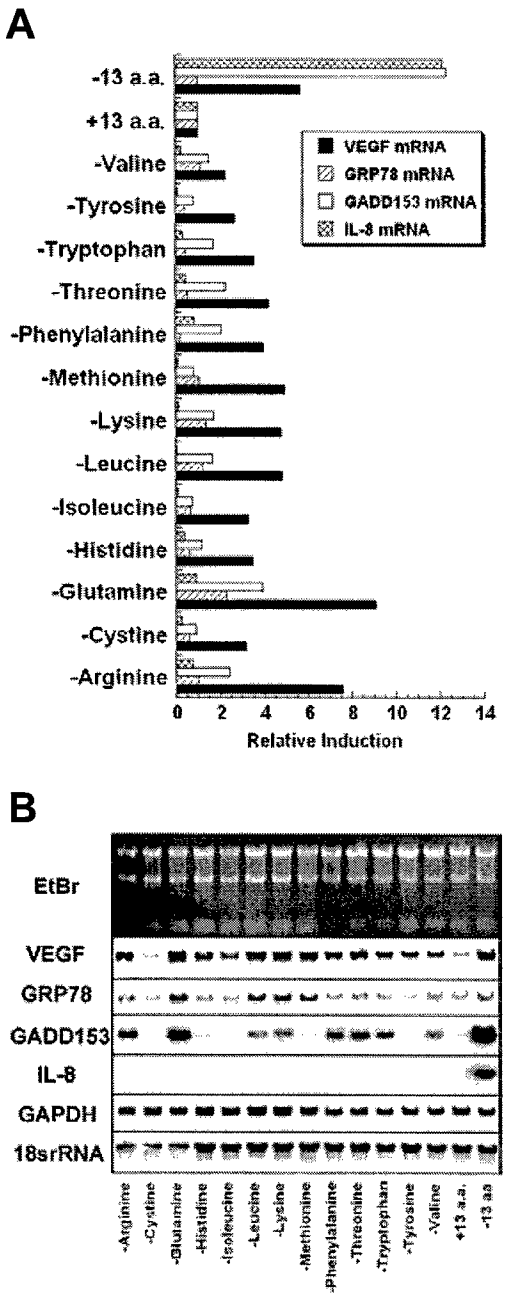

Figure 3
Response of VEGF, IL-8, GRP78, GADD153 and GAPDH mRNA expression to deprivation of various amino acids. TSE cells adapted to MEM were grown to confluence, and cultured for 12 hours in MEM without any amino acids (-13 a.a.), containing the normal supplement of amino acids (+13 a.a.), or in media in which one amino acid at a time was deleted as indicated. Total RNA was isolated and Northern blotting analysis was performed. Fold increases in mRNA levels relative to the sample containing the normal supplement of amino acids (+13 a.a.) are shown in (A).

The kinetics and sensitivity of VEGF, IL-8, GRP78, GADD153, and GAPDH mRNA expression to glutamine deprivation

The effects of glutamine deprivation were further characterized by monitoring the expression of VEGF, IL-8 and ER stress response genes following various durations of complete glutamine starvation and culture conditions with a range of initial media glutamine concentrations. To examine the kinetics of inductions for VEGF, GRP78, GADD153 and IL-8 mRNA expressions, TSE cells were grown to confluence, rinsed twice and fed with glutamine-free DMEM supplemented with dFBS. At various times (0, 3, 6, 12, 24, 48 and 72h) total RNA was isolated and Northern blot analyses for VEGF, GRP78, GADD153, IL-8, GAPDH and 18S rRNA were performed (Figure 4A). The amounts of these mRNAs were determined relative to the amount of 18S rRNA in each sample and compared with the 0 hour time point control. VEGF mRNA levels increased rapidly to 3-fold the initial level by 3 hours and continued to increase, reaching 7-fold at 72 hours of glutamine deprivation. GRP78 mRNA expression was induced 1.5-fold at three hours and, 2.5-fold at 6 hours and maintained about a 2-fold induction for the rest of the time course. GADD153 mRNA expression was induced 3.2-fold at 3 hours and maintained roughly the same level of expression until the 72 hour time point. IL-8 mRNA expression was promptly induced, reaching 4.7-fold by 3 hours and a maximal induction of 15-fold at 6 hours. Following a maximal induction at 6 hours, IL-8 mRNA levels decreased to about 4-fold at 24 hours and maintained this level of expression until the 72 hour time point. GAPDH levels actually decreased slightly during glutamine deprivation.

To determine the sensitivity of VEGF and IL-8 expression to ambient glutamine levels and to compare their expression with the expression of GRP78, GADD153, GAPDH mRNA, TSE cells were grown to confluence, rinsed twice and fed with DMEM containing 0.0, 0.06, 0.125, 0.25, 0.5, 1, 2, or 4 mM glutamine and supplemented with dFBS. The cells were then cultured for 24 hours, total RNA was isolated and Northern blotting analysis was performed (Figure 4B). The amount of VEGF, GRP78, GADD153, IL-8 and GAPDH mRNAs were determined relative to the amount of 18S rRNA in each sample, and these values compared with mRNA contents of the 4 mM glutamine sample. VEGF mRNA expression increased measurably as initial media glutamine concentrations were reduced below 2 mM. At the relatively low glutamine concentration of 250 μM, VEGF mRNA expression was induced 4.7-fold, and was maintained at a 5-fold level until glutamine was totally absent. Thus, VEGF expression was very responsive to a range of glutamine concentrations that compare favorably with the human physiological plasma concentration of 0.6 mM. The dose-response

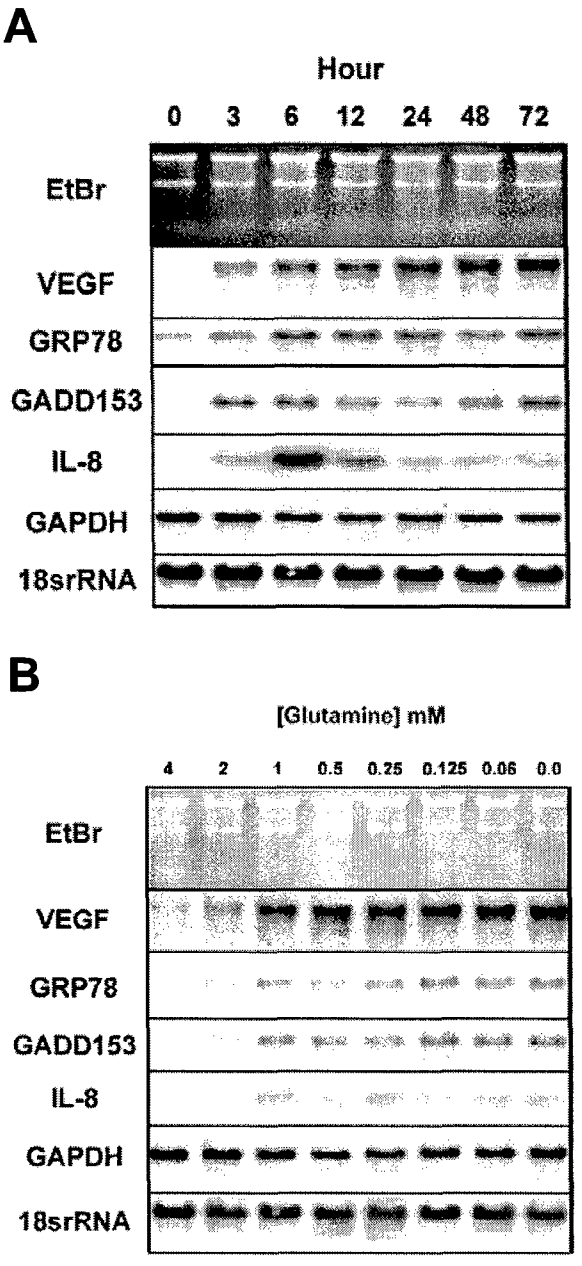

Figure 4
Response of VEGF, IL-8, GRP78, GADD153, and GAPDH mRNA expression to glutamine starvation over time, and to various initial concentrations of glutamine in the media. (A) Confluent TSE cells were cultured in glutamine-free medium for the times indicated, or (B) in media containing the indicated initial concentrations of glutamine for 24 hours. Total RNA was isolated, and Northern blotting analysis was performed.

of inductions for IL-8, GRP78, and GADD153 mRNAs all closely resembled that of VEGF mRNA. Comparison of VEGF and IL-8 mRNA levels gave a correlation coefficient of r = 0.95. Comparison of VEGF with GADD153 mRNA levels yielded a correlation coefficient of r = 0.99. Comparison of VEGF with GRP78 mRNA levels yielded a correlation coefficient of r = 0.90. Thus, VEGF and IL-8 mRNA expression was induced rapidly in response to glutamine starvation, was regulated within a physiologically Relevant range of glutamine concentrations, and correlated strongly with other ER stress-responsive genes. GAPDH mRNA levels did not correlate with those of VEGF.

Discussion

In 1992 Sweiki and co-workers published a landmark paper suggesting that expression of VEGF in response to hypoxia was responsible for neovascularization of glioblastoma multiform [45]. Since then, numerous studies on the regulation of VEGF by hypoxia have been published. The relevance of this regulation was highlighted as it was eventually experimentally demonstrated that rapidly growing tumors are indeed generally hypoxic, with regions of very low oxygen tensions [1,46-48]. Thus, there is now the strong perception that oxygen is the limiting environmental factor in tumor growth and the major factor in controlling tumor VEGF expression and angiogenesis.

However, there are some inconsistencies in the tumor hypoxia-VEGF theory. For example, Kotch and co-workers studied the role of HIF-1 in embryonic growth and vascular development by creating a HIF-1α knockout mouse [11]. Rather than expressing less VEGF as expected, HIF-$1\alpha^{-/-}$ embryos expressed even more VEGF than the HIF-$1\alpha^{+/+}$ or HIF-$1\alpha^{+/-}$ embryos at the same stage. The authors surmised that it was glucose deprivation that had caused up-regulation of VEGF and demonstrated that cultured cells increased VEGF expression in response to glucose deprivation. When Tsuzuki and colleagues used HIF-$1\alpha^{-/-}$ embryonic stem (ES) cells to form tumors, they found that tumor VEGF expression was diminished. However these HIF-$1\alpha^{-/-}$ tumors expressed nearly two-thirds the amount of VEGF that wild-type ES cell tumors expressed [12]. They concluded that some of this VEGF expression was due to tumor-infiltrating stromal cells. However, these HIF-$1\alpha^{-/-}$ tumors expressed significantly more VEGF than tumors formed from $VEGF^{-/-}$ ES cells. Likewise, when Maxwell and co-workers used HIF-$1\beta^{-/-}$ hepatoma cells to form tumors, they found that the expression of the hypoxia-responsive gene GLUT3 was abrogated, but the expression of VEGF was only reduced [13]. In addition, when Griffiths and colleagues [49] compared the vessel density of HIF-$1\beta^{-/-}$ hepatomas to wild type hepatomas they observed "no differences in vascularity." These results were not due to

alternative mechanisms of hypoxic induction of VEGF, for all these studies showed that basal VEGF expression was negligible in HIF-1$\alpha^{-/-}$ or HIF-1$\beta^{-/-}$ cells and that VEGF expression was not induced when these knockouts were made hypoxic *in vitro*. Thus, from these studies we conclude that although hypoxia plays an important role in induction of tumor VEGF expression, other factors also contribute to the control of VEGF expression *in vivo*.

Raliegh and co-workers tested the role of hypoxia in the induction of tumor VEGF expression when they compared the distributions of VEGF protein and the hypoxic marker pimonidazole in eighteen patients with invasive squamous cell carcinoma of the uterine cervix and head and neck [14]. These authors stated that, "a quantitative immunohistochemical comparison of hypoxia and VEGF protein expression revealed no correlation between these two factors." Similarly, Fukumura and colleagues studied the distribution of a hypoxic marker and VEGF expression in tumors and found no spatial correlation [15]. West and co-workers used a needle polarographic oxygen electrode to directly measure oxygen tensions in micro-regions of 38 human cervical cancers prior to tumor removal and then compare these measurements to regional VEGF and platelet-derived endothelial growth factor expression levels measured by immunohistochemistry of tumor sections [16]. These authors stated that "There was no relationship between hypoxia and the expression of angiogenic factors (VEGF, PD-ECGF)." From these data, we conclude that it is unclear to what extent the mechanism of VEGF induction by hypoxia contributes to tumor angiogenesis. We propose that environmental factors other than hypoxia may also contribute to induction of VEGF expression in the tumor environment.

Tumor cells can also be subjected to glucose and amino acid deprivation in the tumor microenvironment. We hypothesized that cancer cells would respond to these forms of nutrient deprivation by increasing the expression of pro-angiogenic factors, such as VEGF and IL-8. This response would allow tumor cells to overcome nutrient deprivation, as well as hypoxia, by increasing the vascular supply of nutrients or by metastasizing to a nutrient rich environment. Using a human breast carcinoma cell line we found that deprivation of glutamine and treatment with tunicamycin caused increased secretion of both VEGF and IL-8. This data suggests that VEGF and IL-8 mRNA continue to be translated and secreted even during a stress, like tunicamycin treatment, that causes ER stress and global translation inhibition. Deprivation of glucose, deprivation of each MEM amino acid except cystine, and specific chemical inducers of ER stress (tunicamycin, Brefeldin A, and A23187) markedly increased the expression of VEGF mRNA. The inductions of VEGF mRNA expression in response to various durations of glutamine deprivation and various glutamine concentrations were well correlated with the inductions of mRNA expression of ER stress-responsive genes GRP78 and GADD153, suggesting that the induction of VEGF expression was also due to a transcriptional ERSR. These studies extend previous findings with other cell types showing that VEGF is responsive to glucose deprivation [11,17-19]. To the best of our knowledge, this is only the second study published that demonstrates that VEGF expression is responsive to amino acid deprivation, the first being our previous study with retinal pigmented epithelial cells [20]. In that study we presented evidence that, similar to hypoxia, glutamine deprivation increased steady-state VEGF mRNA levels through both transcriptional activation and mRNA stabilization.

Treatment with tunicamycin caused greater increases in secreted VEGF and IL-8 protein accumulation during a 24 hour period than did treatment with 0.25 mM, glutamine-containing media. In contrast, the level of VEGF and IL-8 mRNAs at the end of 24 hour period were increased more by glutamine starvation (0 mM) than tunicamycin treatment. In response to tumicamycin, VEGF mRNA increased by 3-fold and secreted protein accumulation increased similarly, by approximately 2-fold. In response to glutamine starvation, IL-8 mRNA increased by 2-fold and secreted protein accumulation increased similarly, by slightly over 2-fold due to less severe glutamine deprivation (0.25 mM). Surprisingly, the accumulation of secreted IL-8 protein occurred with tunicamycin treatment (Figure 1), while the level of IL-8 mRNA in like-treated cells shown in Figure 2A is negligible. However, protein accumulation in the media is expected to be a function of the integrated temporal mRNA level over the 24-hour period. As demonstrated for glutamine deprivation, the temporal nature of the IL-8 response is transient (Figure 4A). Thus the mRNA level at 24 hours should not be expected to be consistent with the protein accumulation. We have not determined the temporal nature of the tunicamycin response.

VEGF mRNA expression by T47D, MCF-7, MDA-MB-453 and MDA-MB-231 breast carcinoma cell lines was also increased in response to nutrient deprivation and chemical inducers of ER stress further suggesting that this is a general phenomenon that occurs in several, if not all, cell lines. Only TSE and MDA-MB-231 expressed IL-8 mRNA levels that were detectable by Northern blotting, and both cells exhibited induction of IL-8 by nutrient deprivation and ER stress. However, chemical inducers of ER stress had quite variable effects on IL-8 expression. It should be noted that hypoxia did not appreciably induce IL-8 expression in any of the breast cancer cell lines tested. This is surprising, given that IL-8 has been identified as being responsive to hypoxia in several tumor cells (for review

see [50]). The hypoxic induction of IL-8 expression is certainly cell-specific, for, in a recent study of a series of transitional cell carcinoma cell lines, Karashima and co-workers found that some cell lines did and some cell lines did not exhibit hypoxic induction of IL-8 expression [51]. It is not the case that 2% oxygen is not severe enough hypoxia to induce IL-8 expression in breast cancer cells, for we have tested total anoxia and not observed IL-8 induction (data not shown).

To the best of our knowledge this is the first demonstration that IL-8 expression is responsive to nutrient deprivation and ER stress. In initial experiments, IL-8 mRNA expression seemed relatively unresponsive to treatments, with the exception of glucose deprivation, A23187 and complete amino acid deprivation. However, the response of IL-8 expression to glutamine deprivation was observed to be substantial at an earlier time than first examined. Because IL-8 transcription is very responsive to NF-κB, which can be stimulated by ER stress [43], it was perhaps predictable that IL-8 expression was induced by ER stress. In contrast, amino acid deprivation would not be expected to activate the UPR or EOR responses, but this also upregulated IL-8 expression. Just recently, Jiang and co-workers showed that amino acid deprivation activates NF-κB, and that this is dependent on phosphorylation of the eukaryotic initiation factor 2 alpha subunit (eIF2alpha) by GCN2 (EIF2AK4) [52]. Thus, it is perhaps also not surprising that amino acid deprivation upregulated IL-8 expression. We have recently found that NF-κB and AP-1 activity is increased by glutamine deprivation of TSE cells and that these factors are necessary for induction of IL-8 expression in response to glutamine deprivation (E. Bobrovnikova-Marjon, unpublished observations). However, a macroarray experiment examining the expression 111 NF-κB-responsive genes found only 13 (including IL-8) that were induced by glutamine deprivation (E. Bobrovnikova-Marjon, unpublished observations). Thus, not all NF-κB-responsive genes are induced by glutamine deprivation.

Glutamine deprivation and chemical inducers of ER stress in conjunction with hypoxia induced the expression of VEGF to a greater extent than hypoxia alone, suggesting that hypoxia and an ERSR pathway can increase the expression of VEGF through distinct mechanisms. In addition, unlike the expression of ER stress-responsive genes, the hypoxia-responsive gene GAPDH was not regulated similarly to VEGF or IL-8. Of interest was the observation that glucose deprivation and hypoxia independently increased the expression of VEGF mRNA, but together had much less effect. A similar phenomena was previously reported to occur in the C6 glial tumor cell line [53]. It is possible that hypoxia combined with glucose deprivation drastically impedes the cell's production of ATP and inhibits the signal transduction processes or transcription factor expression needed for these responses. However, GADD153 mRNA induction was not severely impeded by the same combination of hypoxia and glucose deprivation.

Of all the amino acids tested, glutamine deprivation increased the expression of VEGF and IL-8 by the greatest extent. Glutamine's function as a nitrogen transporter, fuel source, and its high concentration in the blood make it a prime candidate for the study of the effects of nutrient availability on the expression of VEGF. TSE cells are particularly dependent upon glutamine for growth and viability, compared to several other human breast carcinoma cell lines [54]. However, it should be noted that confluent cultures of TSE cells, like those used in the current study, do not exhibit appreciable signs of cell death when glutamine starved for even extended periods (data not shown). We previously observed that tumor xenographs formed from TSE cells contained progressively less glutamine as tumor size increased, demonstrating that glutamine deprivation can occur *in vivo* [55].

The main rationale for this study was to test the hypothesis that nutrient deprivation and/or ER stress increases cancer cell VEGF and IL-8 expression. This is complemented by previous studies suggesting that tumor cells do experience ER stress *in vivo*. Gazit and co-workers demonstrated that the GRP78 promoter was able to drive high expression levels of green fluorescent protein (GFP) in an experimental murine fibrosarcoma tumor model [21]. This group went on to use the GRP78 promoter to drive the tumor-specific expression of HSV-tk as a scheme for cancer gene therapy [22]. Recently this group produced a transgenic mouse containing the mouse GRP94 ER stress-responsive promoter fused to the LacZ reporter gene [23]. This promoter, which contained three ER stress response DNA elements (ERSRE), was inactive in all normal tissues examined but was highly active in both spontaneously-formed and DMBA-induced tumors. Close examinations of these tumors revealed that LacZ expression was greatest in both tumor cells and macrophages at the border of necrotic areas. In addition, several other studies have demonstrated that genes activated in response to ERSR pathways are expressed in tumors. These include GRP78 [24,56], GRP78, GRP94 and calnexin [25].

Conclusions

Recently, there have been a great number of discoveries revealing the role of ERSR in normal development and pathological processes. We contend that the solid tumor environment, with its abnormal and insufficient vasculature, is a prime candidate for ERSR activation and we hypothesize that ERSR can trigger an angiogenic switch in an effort to improve nutritional delivery. Further experi-

ments are needed to determine if ERSR signal transduction pathways and transcription factors are involved in the inductions of VEGF and IL-8 expression by nutrient deprivation and ER stress. Perhaps more importantly, it remains to be determined if these mechanisms are active and contributing to the expression of VEGF and IL-8 and thus the angiogenic and metastatic phenotype of tumor cells *in vivo*.

Methods

Cell Culture

TSE human breast carcinoma cells were provided by Dr. Simon Powell (Massachusetts General Hospital, Department of Radiation Oncology, Boston, MA). T47D, MCF-7, MDA-MB-453 and MDA-MB-231 breast carcinoma cell lines were obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose formulation) supplemented with 4 mM L-glutamine, 10% v/v fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. For Northern blot analyses, cells were plated in 60 cm^2 tissue culture dishes, grown to confluence, fed with fresh medium one day before being rinsed twice with Dulbecco's phosphate-buffered saline (DPBS), and re-fed with fresh media containing drugs or other treatments as described in the figure legends. For glutamine-deprivation experiments, glutamine-free medium (DMEM supplemented with 10% v/v dialyzed FBS (dFBS) and no glutamine) containing added glutamine or drugs was used as described in the figure legends. Experiments including glucose-starved conditions were performed with glucose-free DMEM-based medium supplemented with dFBS as described. Starvation for multiple amino acids was performed with TSE cells adapted to growth over several successive passages in modified Eagle's media (MEM) supplemented as described above for DMEM and dFBS was used during the experiments. Cells subjected to hypoxic stress were grown under normoxic culture conditions until treatment, rinsed twice with DPBS, then treated as described in the legends and placed under hypoxic conditions (37°C, 5% CO_2, 2% O_2, and 95% humidity). For the ELISA, cells were plated in 10 cm^2 wells of 6-well plates, grown to confluence, fed with fresh medium one day before being rinsed twice with DPBS, and re-fed with fresh medium (3 mL per well), containing drugs or other treatments, as described in the figure legend.

ELISA assays

ELISA assays were performed with commercial VEGF and IL-8 ELISA kits (R&D Systems, Minneapolis, MN). Assays for each sample were performed in duplicate, and readings were compared with standard curves obtained with human recombinant $VEGF_{165}$ provided.

RNA isolation and analysis

Total RNA was isolated by the one-step acid-phenol guanidinium procedure [57], with RNA extraction reagent (RNA-Stat60; TelTest, Friendswood, TX), according to the manufacturer's protocol, followed by an additional phenol-chloroform extractions. Northern blot analysis was performed as previously described [58] with cDNAs corresponding to human VEGF (dbEST189750), GRP78 (HAEAC89; ATCC), GADD153 (dbEST298470), IL-8 (dbEST6044688), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, pHcGAP; ATCC) and rat 18S rRNA used as templates to generate ^{32}P-labeled probes with a random-primer labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ). The rat 18S rRNA cDNA template was RT-PCR generated from total rat kidney RNA using R18F2 sense (5'-GCTACCACATCCAAGGAAGGC-3') and R18B1 anti-sense (5'-CCCGTGTTGAGTCAAATTAAGCC-3') primers.

Statistical analyses

Results of Northern blot analyses were quantified using a phosphorescence imager (STORM) and accompanying software (ImageQuant; Molecular Dynamics, Sunnyvale, CA). Integrated band intensities for the probed mRNAs were divided by those for 18S rRNA to obtain normalized values of mRNA levels in each sample. Fold increases were obtained by dividing normalized integrated band intensities for experimental samples by that of a control (untreated, time 0, or complete media) sample. ELISA assays were performed in duplicate, and readings were compared with standard curves obtained with human recombinant $VEGF_{165}$ provided. Means and standard deviations of concentrations in triplicate samples were compared by Student's *t*-test.

Competing interests

None declared.

Authors' contributions

PLM performed all experiments with the assistance of EVB-M and wrote the initial draft of the manuscript. EVB-M assisted with tissue culture, Northern blotting and with composition of figures and text. SFA planned and supervised the study and wrote the final draft of the manuscript.

Additional material

Additional File 1

Supplemental Data Figure S1. VEGF, IL-8, GRP78, GADD153, and GAPDH mRNA expression in MDA-MB-453, T47D, MDA-MB-231, TSE, and MCF-7 cells following 8 h of glutamine deprivation, glucose deprivation or treatments with chemical inducers of ER stress under normoxic and hypoxic conditions. Confluent cells were fed with complete control medium (4Q), glutamine-free medium (-Q), glucose-free medium (-Glucose), medium containing 5 μg/mL tunicamycin, 500 nm Thapsigargin, 10 μM A23187, 1 mM DTT, 10 μg/mL Brefeldin A, or medium containing 0.1% (v/v) DMSO as a vehicle control and cultured under either normoxic (20% O_2) or hypoxic (2% O_2) conditions for 8 hours. Total RNA was isolated and Northern blotting analysis performed. Click here for file [http://www.biomedcentral.com/content/supplementary/1476-4598-3-4-S1.pdf]

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References

1. Vaupel P, Hockel M: **Blood supply, oxygenation status and metabolic micromilieu of breast cancers: characterization and therapeutic relevance.** *International Journal of Oncology.* 2000, **17**:869-879.
2. Giordano FJ, Johnson RS: **Angiogenesis: the role of the microenvironment in flipping the switch.** *Current Opinion in Genetics & Development.* 2001, **11**:35-40.
3. Marti HH, Risau W: **Angiogenesis in ischemic disease.** *Thrombosis & Haemostasis* 1999, **82**:44-52.
4. Harris AL: **Hypoxia--a key regulatory factor in tumour growth.** *Nature Reviews. Cancer.* 2002, **2**:38-47.
5. Ferrara N: **Role of vascular endothelial growth factor in regulation of physiological angiogenesis.** *American Journal of Physiology - Cell Physiology* 2001, **280**:C1358-66.
6. Ferrara N: **Molecular and biological properties of vascular endothelial growth factor.** *Journal of Molecular Medicine* 1999, **77**:527-543.
7. Baek JH, Jang JE, Kang CM, Chung HY, Kim ND, Kim KW: **Hypoxia-induced VEGF enhances tumor survivability via suppression of serum deprivation-induced apoptosis.** *Oncogene* 2000, **19**:4621-4631.
8. Pidgeon GP, Barr MP, Harmey JH, Foley DA, Bouchier-Hayes DJ: **Vascular endothelial growth factor (VEGF) upregulates BCL-2 and inhibits apoptosis in human and murine mammary adenocarcinoma cells.** *British Journal of Cancer* 2001, **85**:273-278.
9. Foekens JA, Peters HA, Grebenchtchikov N, Look MP, Meijer-Van Gelder ME, Geurts-Moespot A, van Der Kwast TH, Sweep CG, Klijn JG: **High tumor levels of vascular endothelial growth factor predict poor response to systemic therapy in advanced breast cancer.** *Cancer Research* 2001, **61**:5407-5414.
10. Gasparini G: **Prognostic value of vascular endothelial growth factor in breast cancer.** *Oncologist* 2000, **5**:37-44.
11. Kotch LE, Iyer NV, Laughner E, Semenza GL: **Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death.** *Developmental Biology* 1999, **209**:254-267.
12. Tsuzuki Y, Fukumura D, Oosthuyse B, Koike C, Carmeliet P, Jain RK: **Vascular endothelial growth factor (VEGF) modulation by targeting hypoxia-inducible factor-1alpha--> hypoxia response element--> VEGF cascade differentially regulates vascular response and growth rate in tumors.** *Cancer Research* 2000, **60**:6248-6252.
13. Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, Ratcliffe PJ: **Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth.** *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**:8104-8109.
14. Raleigh JA, Calkins-Adams DP, Rinker LH, Ballenger CA, Weissler MC, Fowler W. C., Jr., Novotny DB, Varia MA: **Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker.** *Cancer Research* 1998, **58**:3765-3768.
15. Fukumura D, Xavier R, Sugiura T, Chen Y, Park EC, Lu N, Selig M, Nielsen G, Taksir T, Jain RK, Seed B: **Tumor induction of VEGF promoter activity in stromal cells.** *Cell.* 1998, **94**:715-725.
16. West CM, Cooper RA, Loncaster JA, Wilks DP, Bromley M: **Tumor vascularity: a histological measure of angiogenesis and hypoxia.** *Cancer Research* 2001, **61**:2907-2910.
17. Park SH, Kim KW, Lee YS, Baek JH, Kim MS, Lee YM, Lee MS, Kim YJ: **Hypoglycemia-induced VEGF expression is mediated by intracellular Ca2+ and protein kinase C signaling pathway in HepG2 human hepatoblastoma cells.** *International Journal of Molecular Medicine* 2001, **7**:91-96.
18. Satake S, Kuzuya M, Miura H, Asai T, Ramos MA, Muraguchi M, Ohmoto Y, Iguchi A: **Up-regulation of vascular endothelial growth factor in response to glucose deprivation.** *Biology of the Cell* 1998, **90**:161-168.
19. Stein I, Neeman M, Shweiki D, Itin A, Keshet E: **Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes.** *Molecular & Cellular Biology* 1995, **15**:5363-5368.
20. Abcouwer SF, Marjon PL, Loper RK, Vander Jagt DL: **Response of VEGF expression to amino acid deprivation and inducers of endoplasmic reticulum stress.** *Investigative Ophthalmology & Visual Science.* 2002, **43**:2791-2798.
21. Gazit G, Kane SE, Nichols P, Lee AS: **Use of the stress-inducible grp78/BiP promoter in targeting high level gene expression in fibrosarcoma in vivo.** *Cancer Research* 1995, **55**:1660-1663.
22. Gazit G, Hung G, Chen X, Anderson WF, Lee AS: **Use of the glucose starvation-inducible glucose-regulated protein 78 promoter in suicide gene therapy of murine fibrosarcoma.** *Cancer Research* 1999, **59**:3100-3106.
23. Reddy RK, Lu J, Lee AS: **The endoplasmic reticulum chaperone glycoprotein GRP94 with Ca(2+)-binding and antiapoptotic properties is a novel proteolytic target of calpain during etoposide-induced apoptosis.** *Journal of Biological Chemistry.* 1999, **274**:28476-28483.
24. Fernandez PM, Tabbara SO, Jacobs LK, Manning FC, Tsangaris TN, Schwartz AM, Kennedy KA, Patierno SR: **Overexpression of the glucose-regulated stress gene GRP78 in malignant but not benign human breast lesions.** *Breast Cancer Research & Treatment* 2000, **59**:15-26.
25. Chen X, Ding Y, Liu CG, Mikhail S, Yang CS: **Overexpression of glucose-regulated protein 94 (Grp94) in esophageal adenocarcinomas of a rat surgical model and humans.** *Carcinogenesis* 2002, **23**:123-130.
26. Korabiowska M, Cordon-Cardo C, Betke H, Schlott T, Kotthaus M, Stachura J, Brinck U: **GADD153 is an independent prognostic factor in melanoma: immunohistochemical and molecular genetic analysis.** *Histology & Histopathology* 2002, **17**:805-811.
27. Yoshimura T, Matsushima K, Tanaka S, Robinson EA, Appella E, Oppenheim JJ, Leonard EJ: **Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines.** *Proceedings of the National Assembly of Science USA* 1987, **84**:9233-9237.
28. Xie K: **Interleukin-8 and human cancer biology.** *Cytokine & Growth Factor Reviews* 2001, **12**:375-391.
29. De Larco JE, Wuertz BR, Rosner KA, Erickson SA, Gamache DE, Manivel JC, Furcht LT: **A potential role for interleukin-8 in the**

metastatic phenotype of breast carcinoma cells. *American Journal of Pathology* 2001, **158:**639-646.
30. Li A, Dubey S, Varney ML, Dave BJ, Singh RK: **IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis.** *Journal of Immunology.* 2003, **170:**3369-3376.
31. Huang S, Mills L, Mian B, Tellez C, McCarty M, Yang XD, Gudas JM, Bar-Eli M: **Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL8) inhibit angiogenesis, tumor growth, and metastasis of human melanoma.** *American Journal of Pathology.* 2002, **161:**125-134.
32. Masuya D, Huang C, Liu D, Kameyama K, Hayashi E, Yamauchi A, Kobayashi S, Haba R, Yokomise H: **The intratumoral expression of vascular endothelial growth factor and interleukin-8 associated with angiogenesis in nonsmall cell lung carcinoma patients.** *Cancer* 2001, **92:**2628-2638.
33. Kim SJ, Uehara H, Karashima T, McCarty M, Shih N, Fidler IJ: **Expression of interleukin-8 correlates with angiogenesis, tumorigenicity, and metastasis of human prostate cancer cells implanted orthotopically in nude mice.** *Neoplasia (New York)* 2001, **3:**33-42.
34. Rofstad EK, Halsor EF: **Vascular endothelial growth factor, interleukin 8, platelet-derived endothelial cell growth factor, and basic fibroblast growth factor promote angiogenesis and metastasis in human melanoma xenografts.** *Cancer Research* 2000, **60:**4932-4938.
35. Inoue K, Slaton JW, Eve BY, Kim SJ, Perrotte P, Balbay MD, Yano S, Bar-Eli M, Radinsky R, Pettaway CA, Dinney CP: **Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer.** *Clinical Cancer Research* 2000, **6:**2104-2119.
36. Fujimoto J, Sakaguchi H, Aoki I, Tamaya T: **Clinical implications of expression of interleukin 8 related to angiogenesis in uterine cervical cancers.** *Cancer Research.* 2000, **60:**2632-2635.
37. Nurnberg W, Tobias D, Otto F, Henz BM, Schadendorf D: **Expression of interleukin-8 detected by in situ hybridization correlates with worse prognosis in primary cutaneous melanoma.[comment].** *Journal of Pathology.* 1999, **189:**546-551.
38. Retzlaff S, Padro T, Koch P, Oelmann E, Lugering N, Mesters RM, Berdel WE: **Interleukin 8 and Flt3 ligand as markers of advanced disease in primary gastrointestinal non-Hodgkin's lymphoma.** *Oncology Reports.* 2002, **9:**525-527.
39. Orditura M, De Vita F, Catalano G, Infusino S, Lieto E, Martinelli E, Morgillo F, Castellano P, Pignatelli C, Galizia G: **Elevated serum levels of interleukin-8 in advanced non-small cell lung cancer patients: relationship with prognosis.** *Journal of Interferon & Cytokine Research.* 2002, **22:**1129-1135.
40. Hoffmann E, Dittrich-Breiholz O, Holtmann H, Kracht M: **Multiple control of interleukin-8 gene expression.** *Journal of Leukocyte Biology.* 2002, **72:**847-855.
41. Takaya H, Andoh A, Shimada M, Hata K, Fujiyama Y, Bamba T: **The expression of chemokine genes correlates with nuclear factor-kappaB activation in human pancreatic cancer cell lines.** *Pancreas* 2000, **21:**32-40.
42. Huang S, DeGuzman A, Bucana CD, Fidler IJ: **Level of interleukin-8 expression by metastatic human melanoma cells directly correlates with constitutive NF-kappaB activity.** *Cytokines, Cellular & Molecular Therapy* 2000, **6:**9-17.
43. Pahl HL, Baeuerle PA: **The ER-overload response: activation of NF-kappa B.** *Trends in Biochemical Sciences* 1997, **22:**63-67.
44. Yuan Y, Beitner-Johnson D, Millhorn DE: **Hypoxia-inducible factor 2alpha binds to cobalt in vitro.** *Biochemical & Biophysical Research Communications* 2001, **288:**849-854.
45. Shweiki D, Itin A, Soffer D, Keshet E: **Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis.** *Nature* 1992, **359:**843-845.
46. Vukovic V, Haugland HK, Nicklee T, Morrison AJ, Hedley DW: **Hypoxia-inducible factor-1alpha is an intrinsic marker for hypoxia in cervical cancer xenografts.** *Cancer Research.* 2001, **61:**7394-7398.
47. Richard DE, Berra E, Pouyssegur J: **Angiogenesis: how a tumor adapts to hypoxia.** *Biochemical & Biophysical Research Communications* 1999, **266:**718-722.
48. Brown JM, Giaccia AJ: **The unique physiology of solid tumors: opportunities (and problems) for cancer therapy.** *Cancer Research.* 1998, **58:**1408-1416.
49. Griffiths JR, McSheehy PM, Robinson SP, Troy H, Chung YL, Leek RD, Williams KJ, Stratford IJ, Harris AL, Stubbs M: **Metabolic changes detected by in vivo magnetic resonance studies of HEPA-1 wild-type tumors and tumors deficient in hypoxia-inducible factor-1beta (HIF-1beta): evidence of an anabolic role for the HIF-1 pathway.** *Cancer Research.* 2002, **62:**688-695.
50. Shi Q, Xiong Q, Le X, Xie K: **Regulation of interleukin-8 expression by tumor-associated stress factors.** *Journal of Interferon & Cytokine Research* 2001, **21:**553-566.
51. Karashima T, Sweeney P, Kamat A, Huang S, Kim SJ, Bar-Eli M, McConkey DJ, Dinney CP: **Nuclear factor-kappaB mediates angiogenesis and metastasis of human bladder cancer through the regulation of interleukin-8.** *Clinical Cancer Research* 2003, **9:**2786-2797.
52. Jiang HY, Wek SA, McGrath BC, Scheuner D, Kaufman RJ, Cavener DR, Wek RC: **Phosphorylation of the alpha subunit of eukaryotic initiation factor 2 is required for activation of NF-kappaB in response to diverse cellular stresses.** *Molecular and cell Biology* 2003, **23:**5651-5663.
53. Shweiki D, Neeman M, Itin A, Keshet E: **Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficiency in multicell spheroids: implications for tumor angiogenesis.** *Proceedings of the National Academy of Sciences of the United States of America* 1995, **92:**768-772.
54. Collins CL, Wasa M, Souba WW, Abcouwer SF: **Determinants of glutamine dependence and utilization by normal and tumor-derived breast cell lines.** *Journal of Cell Physiology* 1998, **176:**166-178.
55. Collins CL, Wasa M, Souba WW, Abcouwer SF: **Regulation of glutamine synthetase in human breast carcinoma cells and experimental tumors.** *Surgery* 1997, **122:**451-63; discussion 463-4.
56. Song MS, Park YK, Lee JH, Park K: **Induction of glucose-regulated protein 78 by chronic hypoxia in human gastric tumor cells through a protein kinase C-epsilon/ERK/AP-1 signaling cascade.** *Cancer Research* 2001, **61:**8322-8330.
57. Chomcynski P, Sacchi N: **Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.** *Anal. Biochem.* 1987, **162:**156-159.
58. Abcouwer SF, Schwarz C, Meguid RA: **Glutamine deprivation induces the expression of GADD45 and GADD153 primarily by mRNA stabilization.** *J. Biol. Chem.* 1999, **274:**28645-28651.

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Homocysteine Increases the Expression of Vascular Endothelial Growth Factor by a Mechanism Involving Endoplasmic Reticulum Stress and Transcription Factor ATF4*

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Vascular endothelial growth factor (VEGF) plays a key role in the development and progression of diabetic retinopathy. We previously demonstrated that amino acid deprivation and other inducers of endoplasmic reticulum-stress (ER stress) up-regulate the expression of VEGF in the retinal-pigmented epithelial cell line ARPE-19. Because homocysteine causes ER stress, we hypothesized that VEGF expression is increased by ambient homocysteine. DL-Homocysteine-induced VEGF expression was investigated in confluent ARPE-19 cultures. Northern analysis showed that homocysteine increased steady state VEGF mRNA levels 4.4-fold. Other thiol-containing compounds, including L-homocysteine thiolactone and DTT, induced VEGF expression 7.9- and 8.8-fold. Transcriptional run-on assays and mRNA decay studies demonstrated that the increase in VEGF mRNA levels was caused by increased transcription rather than mRNA stabilization. VEGF mRNA induction paralleled that of the ER-stress gene *GRP78*. Homocysteine treatment caused transient phosphorylation of eIF2α and an increase in ATF4 protein level. Overexpression of a dominant-negative ATF4 abolished the VEGF response to homocysteine treatment and to amino acid deprivation. VEGF mRNA expression by ATF4−/− MEF did not respond to homocysteine treatment and the response was restored with expression of wild-type ATF4. These studies indicate that expression of the pro-angiogenic factor VEGF is increased by homocysteine and other thiol-containing reductive compounds via ATF4-dependent activation of VEGF transcription.

Vascular endothelial growth factor (VEGF)[1] is a secreted glycoprotein with endothelial cell-specific mitogenic properties and causes permeability changes in endothelial cell layers (1). VEGF has a distinct role in physiologic angiogenesis and is thus essential for normal embryogenesis (2). Regulation of VEGF expression is involved in many angiogenesis-driven pathologies including diabetic retinopathy (DR), a leading cause of morbidity among diabetic patients and the leading cause of new blindness for persons of working age (3). DR is characterized by loss of retinal capillaries leading to progressive retinal ischemia, increased retinal vascular permeability, and new retinal vessel growth. During proliferative stages of DR plasma and vitreous levels of VEGF are elevated in diabetic patients (4). Many different cell types in the eye produce VEGF, including retinal pigment epithelium, endothelial cells, pericytes, glial cells, and ganglion cells (5).

An understanding of the stimuli that up-regulate VEGF expression in the diabetic retina is essential to development of effective treatments for DR. Several theories have been proposed to explain the VEGF-driven neovascularization of the retina. Oxygen tension markedly affects VEGF expression, increasing VEGF transcription and stabilizing VEGF mRNA (6). In addition, VEGF expression is increased by advanced glycation end products (AGE) (7), high glucose (8, 9), glucose deprivation (10), and by exposure to chemical inducers of ER stress (11). Thus, factors other than hypoxia may influence retinal VEGF expression and play a role in triggering the vascular complications associated with diabetes.

Although controversial (12, 13), several studies have shown an association between homocysteinemia and vascular diseases, including the development of DR (14, 15), ocular venous occlusion (16, 17), and neovascular age-related macular degeneration (18). However, a mechanism by which elevated homocysteine could contribute to development of retinal neovascularization is obscure. Moore *et al.* (19) demonstrated that intraocular injection of homocysteine in mice stimulated *N*-methyl-D-aspartate (NMDA) receptors and apoptotic cell death in the retinal ganglion cell layer in a manner that resembled early diabetic degenerative processes. However, these authors did not examine the effect of homocysteine on retinal VEGF expression, permeability or neovascularization. Another effect of homocysteine is perturbation of protein disulfide formation and protein folding within the endoplasmic reticulum (ER) (20,

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; AARE, amino acid response elements; ATF4, activating transcription factor 4; DN, dominant negative; DR, diabetic retinopathy; DTT, dithiothreitol; eIF2α, eukaryotic initiation factor 2α; GADD153, growth arrest and DNA damage inducible gene 153; GFP, green fluorescent protein; GRP78, glucose-regulated protein 78; IRE1, human homologue of the yeast IRE1p inositol prototrophy gene; MEF, mouse embryo fibroblasts; NSRE, nutrient stress response elements; PERK, PKR-like ER kinase; UPR, unfolded protein response; Wt, wild-type; XBP-1, X-box binding protein-1; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle's medium; AS, asparagine synthetase; Hcys, homocysteine.

21). Homocysteine induces ER stress-responsive genes, including *GRP78*, *GADD153*, and *ATF4* (22, 23). Werstuck *et al.* (24) showed that the altered gene expression in homocysteine-treated cells was due to activation the ER stress response pathway known as the unfolded protein response (UPR).

The UPR represents a set of signaling cascades by which conditions within the ER are communicated to the protein translation machinery and to the nucleus in order to balance the folding capacity of the ER with the protein processing demand (25). These cascades are activated when the function of the ER is perturbed, or when the substrate protein burden on the ER outstrips the processing capacity. One branch of the UPR is initiated by activation of PERK, an eIF2α kinase. Phosphorylation of eIF2α inhibits GDP/GTP exchange and thus the re-formation of ternary translation initiation complexes, thereby slowing global protein translation. Decreased initiation paradoxically leads to increased expression of ATF4. The structure of ATF4 mRNA includes a number of short upstream open reading frames (uORF) that precede the functional coding sequence. Phosphorylation of eIF2α creates conditions that favor downstream re-initiation and synthesis of ATF4 in ER-stressed and nutrient-deprived cells (26). Ultimately, ATF4 induces the expression of numerous genes, including genes involved in amino acid import, metabolism, and assimilation (27).

In this study, we demonstrate that homocysteine is a novel inducer of VEGF expression in a human retinal pigmented epithelial cell line (ARPE-19) and that homocysteine increases VEGF expression due to ATF4-dependent activation of VEGF transcription.

EXPERIMENTAL PROCEDURES

Cell Culture—ARPE-19 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM, low glucose formulation) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Mouse embryo fibroblasts (MEF) obtained from homozygous and heterozygous ATF4 knockout mice (28) were maintained in DMEM (high glucose formulation) supplemented with 15% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, and 10 μM β-mercaptoethanol. For Northern blotting experiments, cells were plated in 10-cm tissue culture dishes and grown to confluence. One day prior to treatment, cells were rinsed twice with Dulbecco's phosphate-buffered saline and fed with fresh medium. DL-Homocysteine, D-homocysteine thiolactone, L-homocysteine thiolactone, or dithiothreitol (DTT) (Sigma) were prepared fresh in DMEM and sterilized by filtration before added to the cell cultures. Reduced D- and L-homocysteine were generated as previously described (29). Briefly, D- and L-homocysteine thiolactone were incubated in 5 N NaOH for 5 min at 20 °C, the solutions were then neutralized with HCl, diluted in DMEM, and administered to the cell cultures. For ELISA experiments, 10^5 cells per well were plated in 6-well plates, grown to confluence, fed with fresh medium 1 day prior to rinse with DPBS (2×), and fed with fresh medium (3 ml per well) containing DL-homocysteine or normal medium. For mRNA decay experiments, cells were treated with homocysteine for 3 h, followed by a dose of 5 μg/μl actinomycin D (actD) (Sigma) and then incubated for various times as described in the figure legends. For experiments utilizing adenoviral vectors, subconfluent cultures of ARPE-19 cells were treated with either ATF4 wild-type (Wt), ATF4 mutant (DN), or the empty AdEasy vector (empty). Twenty hours postinfection, the percentage of cells infected was determined from the expression of green fluorescent protein (GFP). Anoxic conditions were created by sealing cell cultures within plastic bags along with a filter paper bag containing finely divided iron (Anaerocult®, Merck, Darmstadt, Germany). Upon addition of water, an oxidative reaction produces an oxygen-free environment confirmed by a colorimetric indicator strip (Anaerotest®, Merck).

Northern Blot Analysis—Northern blotting was performed as previously described (30) using cDNAs corresponding to human VEGF (dbEST189750), GADD153 (dbEST 298470), GRP78 (HAEAC89, ATCC), and asparagine synthetase (BI752279 IMAGE Clone), and normalized to 18 S rRNA. 18 S rRNA template was generated using mouse RNA and the one-step RNA PCR reagent kit GeneAmp® Gold RNA PCR Reagent kit (Applied Biosystems, Foster City, CA) with the following primers (5′-GCTACCACATCCAAGGAAGGC-3′) and (5′-CCCGTGTTGAGTCAAATTAAGCC-3′). Total RNA was isolated by the one-step acid-phenol guanidinium procedure (31) utilizing RNA-Stat60™ Reagent (TelTest, Friendswood, TX) according to the manufacturer's protocol followed by an additional acid-phenol, phenol/chloroform/isoamyl alcohol, chloroform extraction, and ethanol precipitation in the presence of 0.1 volumes of 3 M sodium acetate. Total RNA (12 μg/lane) was fractionated on 0.2 M formaldehyde/1.0% (w/v) agarose gels and transferred overnight onto a 0.45 micron Magna nylon membrane (Osmonics, Westborough, MA) in 10× SSC. The RNA was cross-linked using a CL-1000 UV cross-linker (UVP, Upland, CA) before hybridization. Specific probes were generated by labeling the cDNA fragments with [α-^{32}P]dCTP (PerkinElmer Life Sciences) using a random primer DNA labeling kit (Amersham Biosciences). Membranes were hybridized with radiolabeled DNA probe for 6–8 h at 60 °C in a solution containing 7% (v/v) SDS, 0.25 M Na_2HPO_4, pH 7.2, as described elsewhere (32). Blotting results were quantified by overnight exposure to a phosphor screen followed by analysis using a STORM™ phosphorimager and Image Quant™ software (Molecular Dynamics, Sunnyvale, CA). For each sample, hybridization to 18 S rRNA was used to normalize results for mRNAs. Fold-inductions were determined by dividing normalized mRNA band intensity volumes for experimental samples to that of control (untreated or time 0) samples. RNA decay analysis was performed using the single compartmental model (see Ref. 33 for review of this model and its application to mRNA decay curves). mRNA half-life was calculated as $\ln(2)/k$, where k is the decay constant estimated by analysis of kinetic data. Decay constants used to calculate half-lives of VEGF mRNAs were estimated by fitting semi-log plots of (corrected, normalized mRNA concentration) *versus* time (30).

RT-PCR Analysis—Complementary DNA (cDNA) was synthesized from 100 ng of total RNA using a RNA PCR reagent kit GeneAmp ® Gold RNA PCR Reagent kit. Polymerase chain reaction (PCR) amplification was performed under the following conditions: 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min; 40 cycles, followed by a 4-min 72 °C incubation performed in a DNA thermal cycler (PerkinElmer Life Sciences). The PCR products were size-fractionated by agarose gel electrophoresis using 3.3% Synergel® (Diversified Biotech, Boston, MA) according to the manufacturer's recommendations and stained with 0.5 μg/ml ethidium bromide. Products were photographed under UV light using the GENEGNOME® imaging system (Synergene, Cambridge, UK). Forward and reverse primers used for the amplification of the edited segment of XBP-1 mRNA were 5′-GAAGCCAAGGGGAATGAAGTGAGG-3′ and 5′- CATGGGGAGATGTTCTGGAGGGG-3′, respectively.

Nuclear Run-on Assay—Triplicate 15 cm plates were treated as described in the figure legend. 3 h post-treatment cells were mechanically removed from flasks by scraping in cold phosphate-buffered saline. Cells were pelleted at 500 × g for 5 min at 4 °C. Cell pellets were resuspended in 4 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40), gently vortexed, and incubated on ice for 10 min. Nuclei were pelleted and washed once with cold lysis buffer. Nuclear pellets were resuspended in 130 μl of RNase free water along with 1 μl of Rnasin and 150 μl of 2× reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 20 μM ATP, 1 mM CTP, 1 mM GTP, and 1 mM UTP). Transcription was initiated by addition of 10 μl of 10 mCi/ml [α-^{32}P]ATP. Nuclei were then incubated in a 30 °C water bath for 30 min. The reaction was then terminated by addition of 50 μl of DNase I buffer (60 mM Tris-HCl, 3.0 M NaCl, 30 mM $MgCl_2$, 12 mM $CaCl_2$) and ~600 units of DNase I followed by incubation for 10 min at 30 °C. Protein digestion was then carried out at 42 °C for 30 min with the addition of 20 μl (5% (w/v) SDS, 0.5 M Tris-HCl, pH 7.4, 0.125 M EDTA), 10 μl of 20 mg/ml proteinase K (200 μg/sample). Following digestion, the nuclear RNA was extracted with the RNeasy® system (Qiagen, Valencia, CA) according to the manufacturer's protocol. Labeled nuclear RNA was captured by immobilized, *in vitro* transcribed chloramphenicol acetyl transferase, GAPDH, and VEGF antisense RNAs. The DNA templates were derived by PCR using the following primers to amplify fragments of cDNAs with the addition of T7 promoter sequences (underlined): VEGF (5′-GCCACCACACCATCACCATCG-3′) and (5′-<u>TAATACGACTCACTATAGG</u>GTCTTTCTGTCCGTCTGACCTGGG-3′), GAPDH (5′-TCAACGGATTTGGTCGTATTGGG-3′) and (5′-<u>TAATACGACTCACTATAGG</u>GAGAAAAGGTGGAGGAGTGGGTGTCG-3′), and chloramphenicol acetyl transferase (5′-ACATTTTGAGGCATTTCAGTCAGTTGC-3′) and (5′-<u>TAATACGACTCACTATAG</u>GTCAGCGGCATCAGCACCTTGTCG-3′). 1 μg of each template DNA was then used to synthesize unlabeled antisense RNA probes using MAXIscript™ *in vitro* transcription kit (Ambion, Austin, TX). RNA was then blotted onto nylon membrane as described previously (35). Mem-

branes were blocked for 30 min in 5 ml of ULTRAhyb® (Ambion) at 68 °C. Hybridization with the labeled nuclear RNA (1–3 × 10^6 cpm/ml) was then carried out for 24 h at 68 °C in ULTRAhyb®. Washing of membranes and detection of captured RNA transcripts was performed as described for Northern blotting analysis (above).

ELISA—ELISA assays were performed using commercial VEGF ELISA kits from R&D Systems (Minneapolis, MN). Conditioned media was collected from wells, aliquoted, and stored frozen until assayed. Samples were diluted 10-fold in dilution buffer provided with the kit prior to assaying. Assays were performed in triplicate, and the readings were compared with standard curves obtained with human recombinant $VEGF_{165}$, provided with the kit.

ATF4 Adenoviral Vector Construction—Wild-type and DN mutant ATF4 cDNAs were expressed using the AdEasy adenoviral vector system (36) provided by Bert Vogelstein (Howard Hughes Institute, Johns Hopkins University). Plasmids containing murine wild-type ATF4 (Wt) cDNA and DN mutant ATF4 (DN) cDNA were kindly provided by Jawed Alam (Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center) who generated the ATF4 Wt plasmid (pEFmATF4myc) by inserting the murine ATF4 cDNA into the PmlI site of the pEF/myc/mito vector (Invitrogen), thus removing the mitochondrial targeting sequence (37). These authors constructed the ATF4ΔRK (DN) plasmid (pEF/mATF4M) by PCR-generated overlap extension to create seven amino acid substitutions within the DNA-binding domain (^{292}RYRQKKR^{298} to ^{292}GYLEAAA^{298}). The mutant cDNA was cloned into the PmlI-NotI sites of the pEF/myc/mito vector, again removing the mitochondrial targeting sequence. To clone into the AdEasy system, Wt and DN coding sequences from these vectors were amplified by PCR using a 5′-NotI-containing (underlined) forward primer (5′-AACAACAACGCGGCCGCTGTCGTGAACACCATGACCGAG-3′) and a 5′-HindIII-containing (underlined) reverse primer (5′-GTTGTTGTTAAGCTTTAGACTATGCGGCCCCATTCAG-3′). The PCR reaction was carried out with Taq polymerase (Applied Biosystems) under the following conditions: 95 °C 1 min, 55 °C 2 min, 72 °C 1 min, for 35 cycles followed by a 7-min 72 °C incubation. The PCR products were cloned into the pAdTrack-CMV adenoviral shuttle vector encoding kanamycin resistance and containing a second expression cassette encoding GFP. The shuttle vectors were then linearized with the restriction enzyme PmeI and electroporated into DH5α electrocompetent cells along with the adenoviral backbone plasmid pAdEasy-1. Clones containing recombinant plasmids pAE-ATF4-Wt and pAE-ATF4-DN, formed by homologous recombination, were subsequently selected for kanamycin resistance and identified by plasmid size in conjunction with endonuclease analysis. At this stage the coding sequences and insertion points of pAE-ATF4-Wt and pAE-ATF4-DN cDNAs were confirmed by the University of New Mexico School of Medicine DNA Sequencing Facility. Recombinant adenoviral vectors were generated by transfecting the 293 packaging cell line with PacI-linearized pAE-ATF4-Wt and pAE-ATF4-DN. Transfected cultures were maintained until the percentage of cells exhibiting green fluorescence approached 100%. Viruses were obtained by freeze/thaw lysis of the cells in phosphate-buffered saline, followed by clarification of the lysates by centrifugation at 6,000 × *g* at 4 °C for 10 min. Lysates were used to infect large scale cultures of 293 cells for production of viral stock as described above.

Western Blot Analysis of ATF4 Production—Cells were grown to confluence in 12-well plates. ATF4 virus was serially diluted from 1:20 to 1:160 in serum-free medium to determine the protein expression of the virus in the ARPE-19 cell line. 24 h following addition of the virus, cells were scraped from the dishes and lysed in ice-cold lysis buffer (0.1% SDS, 0.5% SDC, 1% Triton X-100, 50 mM Tris-HCl, pH 8.0). Protein contents of cleared lysates were determined with a BCA Protein Assay Kit (Pierce), and equal amounts of proteins (40 μg) were loaded into each lane and separated on a 10% SDS-polyacrylamide (SDS-PAGE) gel. The protein bands were then transferred to a nitrocellulose membrane (Bio-Rad) and probed with antibodies specific for ATF4 (Santa Cruz Biotechnology), phosphorylated eIF2α (Cell Signaling Technology, Beverly, MA), or β-actin (Sigma). Proteins were detected with ECL™ chemiluminescence kit (Amersham Biosciences) according to the manufacturer's instructions. Membranes were then scanned and viewed by MultiGenius Bioimaging System® (Cambridge, UK).

RESULTS

Response of VEGF, GADD153, and GRP78 mRNA Expression in ARPE-19 Cells to Homocysteine and other Thiol Compounds—To test the hypothesis that expression of VEGF mRNA is responsive to homocysteine and reductive stress, confluent ARPE-19 cells were cultured for 4 h in the following: 1) normal medium; 2) medium containing 10 mM DL-homocysteine; 3) medium containing 10 mM L-cysteine; 4) medium containing 10 mM L-homocysteine thiolactone (the intramolecular ring counterpart to homocysteine); and 5) medium containing 5 mM DTT (a reducing agent known to cause ER stress). Total RNA was isolated, and normalized mRNA contents for each sample were compared with that of the untreated control (Fig. 1*A*). The ARPE-19 cells contained detectable amounts of a single mRNA species corresponding to VEGF. The amounts of VEGF mRNA relative to 18 S rRNA were increased 4.4-fold by DL-homocysteine, 7.9-fold by L-homocysteine thiolactone, and 8.8-fold by DTT. Cysteine caused an apparent 1.8-fold increase in VEGF mRNA content that was not statistically significant. To differentiate between the D- and L- isomers of homocysteine, D- and L-homocysteine thiolactone were hydrolyzed to produce D- and L-homocysteine, and cells were treated with 10 mM of each compound. Both D- and L-homocysteine were equally capable of inducing VEGF expression (data not shown), suggesting that the effect did not depend upon the cellular metabolism of homocysteine.

The expression of ER stress-responsive genes was also examined to determine if these treatments caused activation of the UPR. The expression of GADD153 was also up-regulated 4.5-fold by DL-homocysteine, 1.9-fold by L-homocysteine thiolactone, and 3.8-fold by DTT. L-cysteine did not significantly up-regulate GADD153 expression compared with the control. In

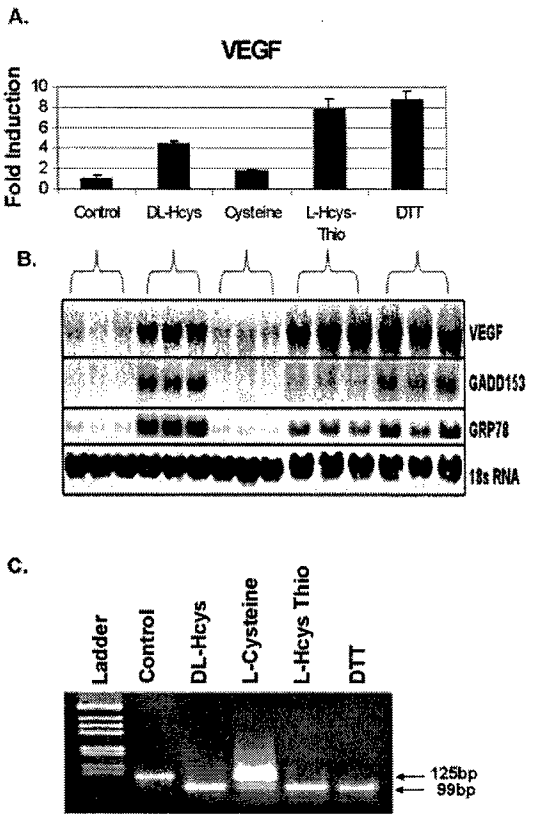

FIG. 1. **Expression of VEGF and ER stress-responsive genes *GRP78* and *GADD153* in response to homocysteine and other thiol containing compounds.** *A*, Northern blotting analysis with coinciding numerical values of relative mRNA inductions. Confluent ARPE-19 cells were cultured for 4 h in normal medium (*Control*), medium containing 10 mM DL-Hcys, medium containing 10 mM cysteine, medium containing 10 mM L-homocysteine thiolactone (*L-Hcys-Thio*), or medium containing 5 mM DTT. *B*, total RNA was isolated and Northern blotting analysis was performed. *C*, RT-PCR analysis of XBP-1 mRNA splicing by IRE1. RT-PCR analysis was performed with primers flanking the XBP-1 mRNA splice site using total RNA. Smaller bands demonstrate the removal of the 26-bp segment by IRE1 endonuclease.

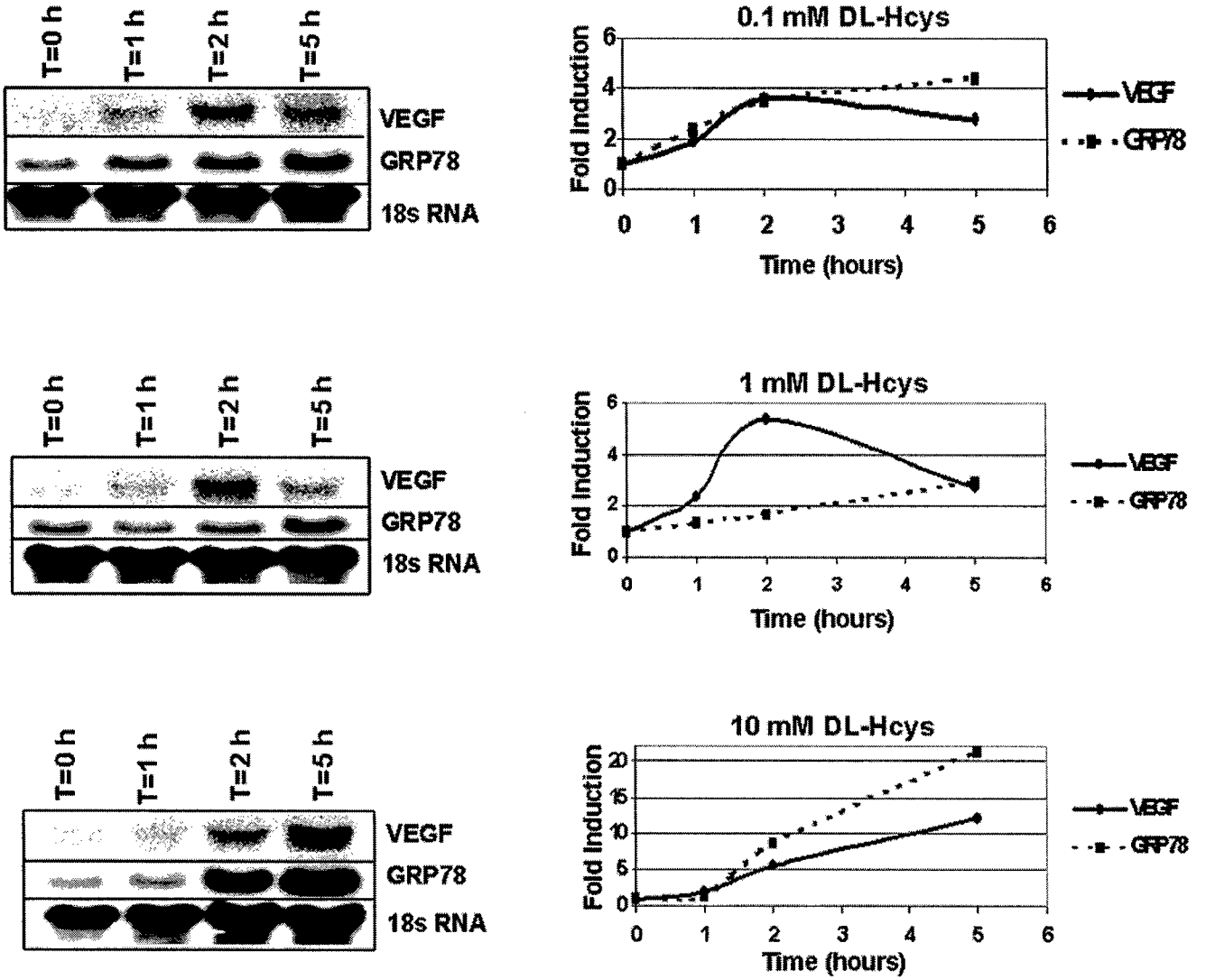

FIG. 2. **Response of VEGF and GRP78 mRNA expression to various concentrations of DL-homocysteine.** Confluent ARPE-19 cells were cultured in 0.1, 1.0, and 10.0 mM DL-homocysteine for the times indicated. Total RNA was isolated, and Northern blotting analysis was performed. Changes in mRNA levels are reported under "Results."

addition, GRP78 was up-regulated 7.3-fold by DL-homocysteine, 3.3-fold by L-homocysteine thiolactone, and 4.8-fold by DTT. L-Cysteine did not significantly affect GRP78 mRNA expression. These results are in good agreement with past studies demonstrating the effect of homocysteine on these genes (22, 23).

The ability of thiol-containing compounds to activate an ER stress response was further evaluated by measuring the splicing of X-box binding protein-1 (XBP-1) mRNA. Upon activation of the UPR, the ER transmembrane protein IRE1 acquires an endonuclease activity that subsequently excises a 26-base pair segment from XBP-1 mRNA, thereby, upon ligation, creating the open reading frame that encodes the transcription factor (38). RT-PCR analysis with primers flanking the splicing site served as an extremely sensitive measure of UPR activation. Fig. 1*B* shows that a large fraction of XBP-1 mRNA is spliced in cells treated with DL-homocysteine, L-homocysteine thiolactone, and DTT. No XBP-1 splicing was detected in the control cells. Treatment with 10 mM cysteine caused only a very small amount of XBP-1 splicing that is illustrated by overloading of that PCR sample.

The Time and Dose Response of VEGF to Homocysteine—To better define the rapidity of this response, a time course study was carried out with hourly measurements from 1 to 5 h in the presence of medium containing 0.1, 1.0, and 10 mM DL-homocysteine (Fig. 2). The time course for 0.1 mM DL-Hcys showed a 2.0-fold increase in VEGF mRNA content within 1 h of treatment. The response to 0.1 mM DL-Hcys was maximal at 2.0 h with a 3.8-fold induction and decreased rapidly thereafter. The up-regulation of GRP78 mRNA expression correlated closely with the up-regulation of VEGF mRNA during the 0.1 mM time course. The time course seen with 1.0 mM showed a similar but more extensive induction of VEGF mRNA, with an apparent increase within 1 h that reached 5.2-fold maximum induction at 2 h, followed by a rapid decline of mRNA levels. The 10 mM homocysteine time course experiment demonstrated an initial VEGF mRNA up-regulation occurring within 1 h and increasing until 5 h, at which time the relative VEGF mRNA level reached 12-fold the initial content. The response to 10 mM homocysteine returned to basal levels at 16 h (data not shown). The time course of GRP78 mRNA response also mirrored the VEGF response in cells treated with 1.0 and 10 mM homocysteine. These data show that the induction of VEGF in response to homocysteine treatment was time-and-dose-dependent and that the up-regulation of an ER stress-responsive gene resembled the induction of VEGF.

Secretion of VEGF by Homocysteine-treated Cells—Global

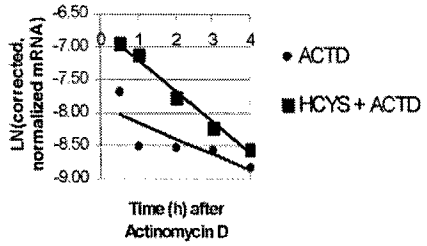

FIG. 3. **Effect of homocysteine on VEGF mRNA decay rate.** Cells were treated for 3 h in the absence or presence of DL-homocysteine, followed by addition of 5 μg/ml actinomycin D. Total RNA was collected at the times indicated and Northern blotting analysis performed. Semi-log graph of normalized relative mRNA levels, corrected by subtraction of the values obtained after 6 h of decay, *versus* time along with lines obtained by linear regression are shown.

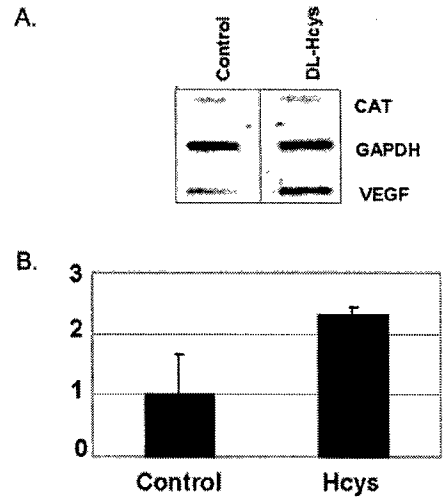

FIG. 4. **Effect of homocysteine on VEGF transcription rate.** *A*, slot blot capture of radiolabeled nuclear RNA. Triplicate 15-cm plates were treated with normal medium (*Control*) or 10 mM DL-homocysteine for 3 h at which time the nuclei were harvested, and the nuclear run on assay was performed. *B*, quantitative analysis of triplicate assays.

protein synthesis is attenuated during the UPR through phosphorylation of the translation initiation factor eIF2α (39). Since VEGF is a glycosylated and secreted protein, its synthesis is dependent on ER function. We therefore tested whether VEGF was secreted during homocysteine-induced ER stress. The levels of VEGF secreted in the medium from cells treated with 10 mM homocysteine were increased 1.3-fold ($p = 0.024$) relative to untreated controls (data not shown). This level of secretion corresponds closely with the previously demonstrated 1.3-fold increased secretion of VEGF observed with glutamine-deprived ARPE-19 cells (11). Although VEGF protein accumulation was significantly increased, it was not proportional to the observed increase of VEGF mRNA levels caused by the same treatment. While this is probably due in part to the transient nature of VEGF mRNA increases during homocysteine treatment, it may also reflect inhibition of translation or protein secretion.

The Effect of Homocysteine on VEGF mRNA Stability—Hypoxia and glutamine deprivation increase VEGF expression by both transcriptional and post-transcriptional mechanisms (11, 40). We investigated the effect of homocysteine on mRNA degradation rate to differentiate between transcription and post-transcriptional effects on VEGF expression. Confluent ARPE-19 cells were treated with 10 mM DL-homocysteine for 3 h, causing VEGF mRNA levels to increase, and then were treated with 5 μg/ml actinomycin D (actD). Control and homocysteine-treated cells were harvested at 0.5, 1, 2, 3, 4, and 6 h after actD treatment and the decline of VEGF mRNA levels were analyzed by Northern blotting (Fig. 3). Decay constants of 0.24 h^{-1} ($r = 0.80$) and 0.48 h^{-1} ($r = 0.99$) were obtained for VEGF mRNA decay in control and treated cells, respectively, suggesting that homocysteine did not cause stabilization of VEGF mRNA. In fact, there was an apparent destabilization of VEGF mRNA upon homocysteine treatment. Although the basal VEGF decay rate obtained agrees with previous reports from other cell types (41–43), it should be noted that accurate measurement of VEGF mRNA decay in untreated cells was difficult due to the low basal level. Regardless, it appeared that mRNA stabilization could not account for the increase of VEGF mRNA levels produced by homocysteine. This suggests that homocysteine causes transcriptional activation of the *VEGF* gene.

Transcriptional Up-regulation of VEGF—To further investigate the role of transcription in the up-regulation of VEGF mRNA a nuclear run-on assay was conducted in ARPE-19 cells treated with either normal medium or 10 mM DL-homocysteine. The slot-blot capture of the radiolabeled nuclear RNA showed that homocysteine increased the transcription rate of VEGF (Fig. 4*A*). Normalization to GAPDH showed a 2.3-fold ($p = 0.03$) increase in VEGF transcription (Fig. 4*B*). The chloramphenicol acetyl transferase RNA was used as a control for nonspecific binding. As shown in Fig. 4*A* there is very little nonspecific binding in the assay. The results demonstrate increased transcriptional initiation of the *VEGF* gene, which could implicate a transcription factor that is ER stress responsive.

eIF2α Phosphorylation and ATF4 Protein Expression in ARPE-19 Cells—Two previous studies reported that the expression of ATF4 mRNA was induced by homocysteine treatment (22, 23). Computer analysis of the human VEGF promoter region sequence revealed the presence of four amino acid response elements (AARE) within reasonable proximity of the transcription start site (data not shown). Because AARE are bound and activated by transcription factor complexes containing ATF4, we hypothesized that this factor contributes to the activation of VEGF transcription in response to homocysteine. Therefore, the effects of 10 mM homocysteine on phosphorylation of eIF2α and ATF4 protein levels were analyzed. Phosphorylation of eIF2α following homocysteine treatment was rapid and transient (Fig. 5*A*). Greatly increased levels of phosphorylated eIF2α protein was detected following 5 min of treatment and returned to basal levels within 60 min. An immunoreactive band corresponding to ATF4 protein was detectable at 60 min and continued to increase in intensity through 240 min (Fig. 5*B*). (The upper and lower bands detected by this antibody were determined to be nonspecific binding as demonstrated below.) These data indicate that homocysteine-treated ARPE-19 cells respond by attenuating protein synthesis through eIF2α phosphorylation resulting in increased ATF4 protein translation. Thus, ATF4 is available to activate *VEGF* transcription.

The Role of ATF4 in the Expression of VEGF in ARPE-19 Cells—To examine the role of ATF4 in the up-regulation of VEGF expression, the AdEasy adenoviral vector system was used to express dominant-negative (ATF4 DN) and wild-type (ATF4 Wt) proteins (37). First, the effects of various amounts of Wt and DN mutant ATF4 adenovector stocks (1:20 to 1:160 dilutions) on the intracellular level of ATF4 antibody-reactive protein were examined at 20-h postinfection (Fig. 6*A*). Viral infection was assessed by determining the percentage of cells that exhibited GFP fluorescence. ATF4 expression was evaluated by Western blotting analysis. Both of the exogenously produced ATF4 proteins were antigenic. Although infection rates were comparable, the expression of the ATF4 DN mutant was approximately twice that of the ATF4 Wt protein at each viral stock dilution. Exogenously produced proteins were expressed at levels much higher than those of the endogenous ATF4. A titration curve for the empty virus was determined by

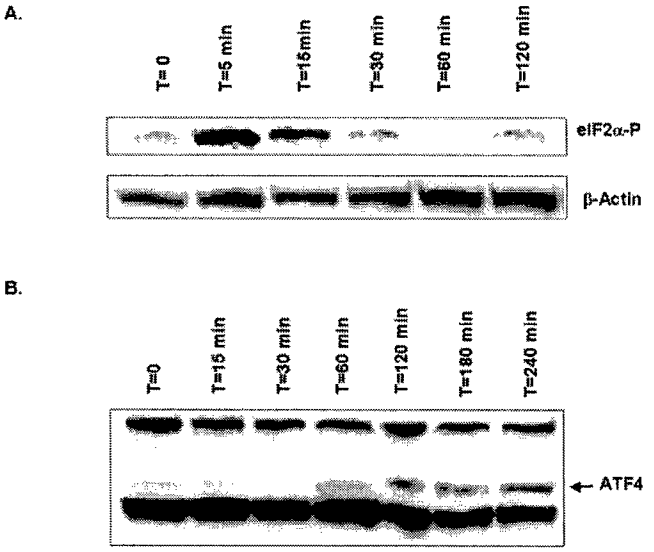

FIG. 5. **Effect of homocysteine on eIF2α phosphorylation and ATF4 protein expression.** *A*, Western blot analysis of eIF2α phosphorylation (*eIF2α-p*) in ARPE-19 whole cell lysates. Cells were treated with 10 mM DL-homocysteine for the indicated times. *B*, Western blot analysis of ATF4 protein levels in ARPE-19 whole cell lysates. Cells were treated with 10 mM DL-homocysteine for the indicated times.

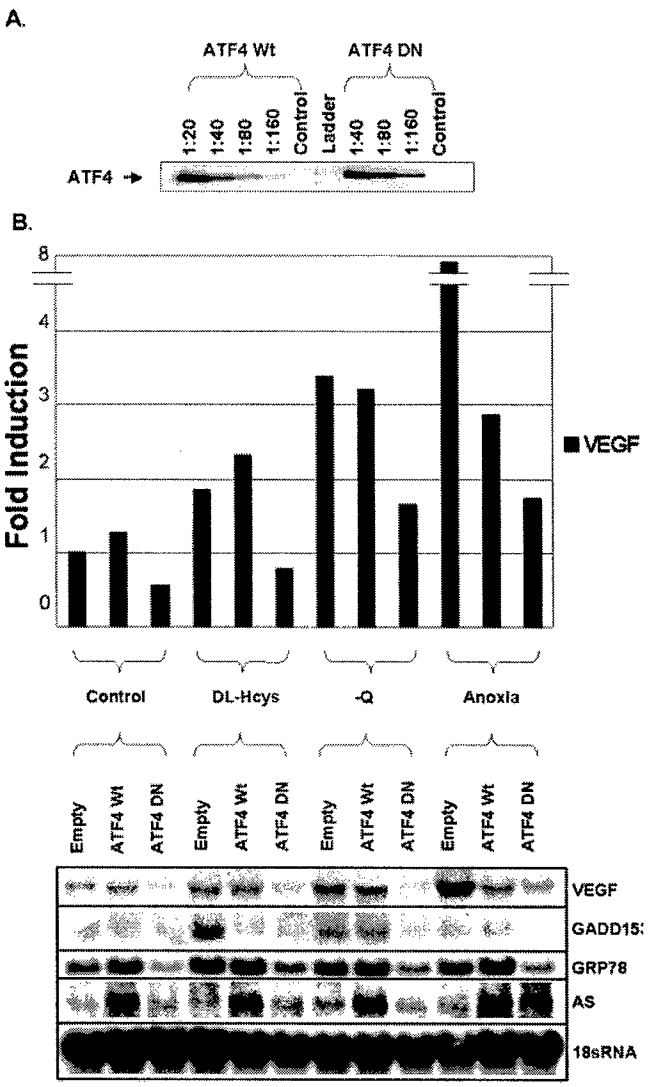

FIG. 6. **Effects of overexpression of wild-type and DN mutant forms of ATF4 on VEGF expression.** *A*, Western blotting analysis of ATF4 proteins in lysates of cells infected with serial dilutions of the ATF4 Wt and DN virus stocks. Subconfluent cultures of ARPE-19 cells were infected with WT or DN virus and 20-h postinfection cells were harvested, and Western blotting with anti-ATF4 antibodies was performed. *B*, Northern blotting analysis of subconfluent cultures of ARPE-19 cells infected with empty, Wt, or DN viruses. 20-h postinfection cultures were treated with normal medium (*Control*), medium containing 10 mM DL-homocysteine, glutamine-free medium, or normal medium in an oxygen-free environment (*Anoxia*). After 4–5 h of treatment, total RNA was isolated, and Northern blotting analysis was performed.

evaluating the percentage of cells exhibiting GFP fluorescence 20-h postinfection. Viral infection of ARPE-19 cells with 1:40, 1:80, and 1:80 dilutions respectively of Wt, DN, and empty virus resulted in infection rates >90% for each (data not shown).

To evaluate the role of ATF4 function in the induction of VEGF expression by various stresses, ARPE-19 cells were infected with empty, ATF4 Wt, and ATF4 DN adenovectors. 20 h after contact with the viruses, cells were fed with fresh medium, and then cells from each virus treatment group were subjected to the following: no treatment (incubated with control medium for 5 h), incubated with medium containing 10 mM DL-homocysteine for 4 h, incubated with medium containing no glutamine (−Q) for 5 h, or incubated in normal medium but subjected to anoxia for 5 h. VEGF mRNA expression was then analyzed by Northern blotting (Fig. 6). VEGF mRNA was up-regulated by DL-homocysteine, nutrient deprivation (−Q), and anoxia. Compared with cells infected with empty vector, the expression of Wt ATF4 increased VEGF mRNA levels in the control cells and had little effect on VEGF levels in DL-homocysteine-treated and glutamine-starved cells. In contrast, the expression ATF4 DN greatly inhibited VEGF expression under all conditions, including anoxia. However, under anoxic conditions the response was somewhat different, in that both the Wt and DN proteins greatly reduced VEGF expression. These results indicate that ATF4 positively affects VEGF mRNA expression, as would be expected if this factor activates VEGF transcription. In addition, ATF4 function is necessary for induction of VEGF expression by homocysteine and glutamine deprivation. However, because both Wt and DN ATF4 protein diminish induction of VEGF mRNA expression during anoxia, it is most likely that these proteins are sequestering an ATF4 binding partner that plays a role in hypoxic induction of this gene.

The expression ATF4 Wt slightly increased the expression of GADD153 mRNA in the control cells. The induction of GADD153 expression in response to homocysteine, nutrient deprivation, or anoxia was inhibited by the expression of ATF4 DN. Surprisingly, both the Wt and DN viruses blocked induction of GADD153 by DL-homocysteine. *GRP78* was recently shown to be an ATF4-responsive gene (44). These data confirm the recent findings of Luo *et al.* (44) using these same adenoviral vectors. That study demonstrated that ATF4 Wt protein expression increased GRP78 mRNA expression and ATF4 DN expression repressed GRP78 expression under all treatment conditions (44). Asparagine synthetase (*AS*), a gene known to be ATF4 responsive (45), was greatly induced by the Wt virus in all treatment groups. Unexpectedly, both Wt and DN ATF4 induced AS under anoxic conditions.

VEGF Expression in ATF4−/− MEF—To further confirm the ATF4 DN results, the effect of homocysteine on VEGF expression was examined in MEF cultures obtained from homozygous (−/−) and heterozygous (+/−) ATF4 knockout mice. The absence of ATF4 was demonstrated in ATF4−/− MEF via Western blotting (Fig. 7*A*). Again, this antibody detected two nonspecifically immunoreactive bands corresponding to the bands observed with ARPE-19 lysates (Fig. 5*A*). However, only in lysates from

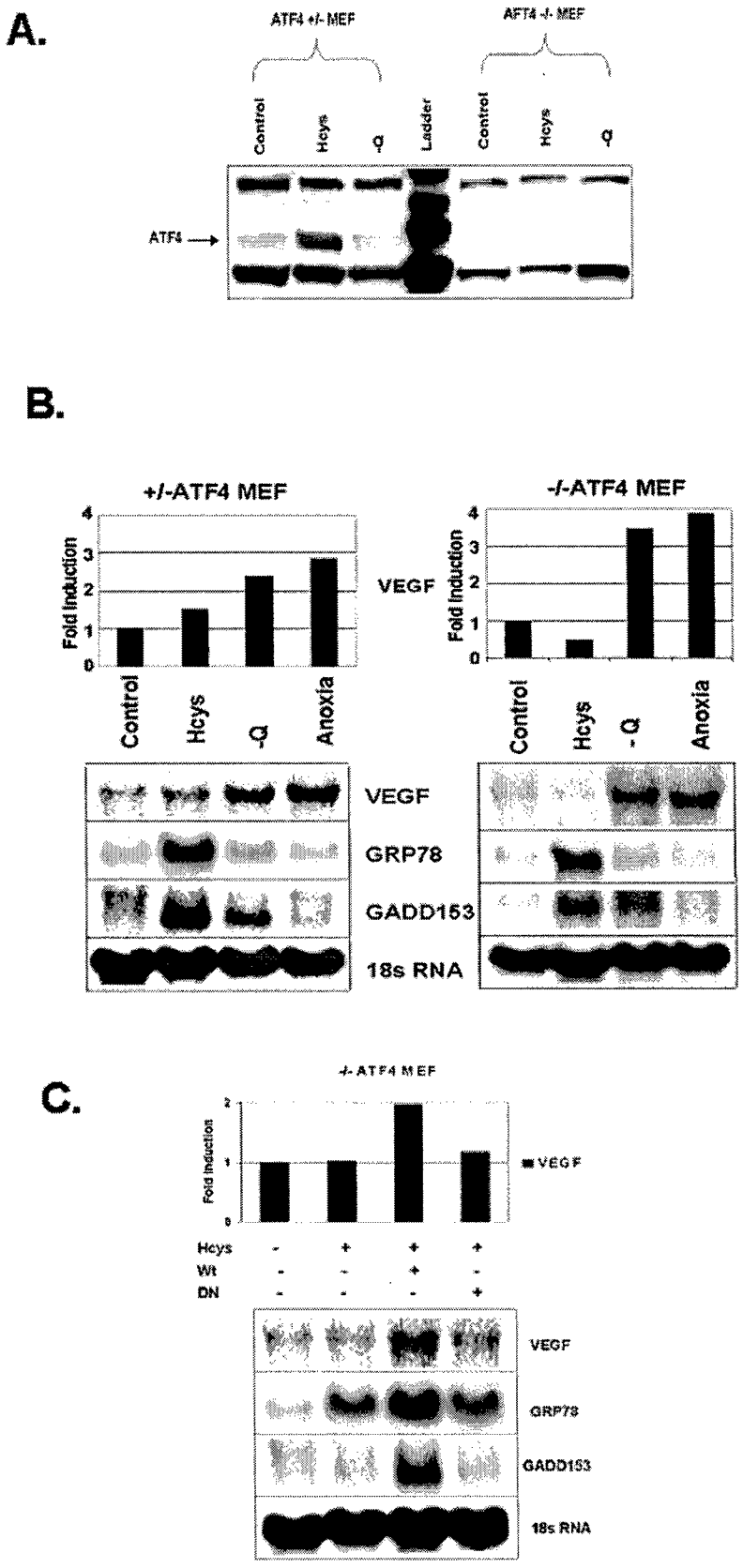

FIG. 7. **Effects of homocysteine treatment and nutrient deprivation on VEGF expression in ATF4−/− MEF.** *A*, Western blot analysis of ATF4 expression in DL-Hcys treated and glutamine (−Q)-starved cells in ATF4+/− and ATF4−/− MEF. MEF were treated with 10 mM DL-homocysteine (4 h) or glutamine starved for 5 h. *B*, Northern blotting analysis of VEGF, GRP78, and GADD153 expression in MEF. MEF were treated with either 10 mM DL-homocysteine (4 h), glutamine-starved (5 h), or anoxia (5 h). Fold inductions of VEGF mRNA are shown. *C*, Northern blotting analysis of VEGF, GRP78, and GADD 153 mRNAs in ATF4−/− MEF. Cells received no treatment (control), 10 mM DL-homocysteine (4 h), homocysteine + ATF4 Wt, or homocysteine + ATF4 DN. Fold inductions of VEGF mRNA are shown.

ATF4+/− MEF was a band of the expected size of ATF4 protein detected. Both MEF cell types were treated with either 10 mM DL-homocysteine for 4 h or glutamine-starved for 5 h. The band corresponding to ATF4 greatly increased in intensity with homocysteine treatment, but was not increased with glutamine deprivation.

To determine the role of ATF4 in VEGF expression, the treatments described in Fig. 6 were repeated with the

ATF4+/− and ATF4−/− MEF cells. ATF4+/− MEF had a higher basal level of VEGF mRNA compared with the ATF4−/− MEF (Fig. 7*B*). The ATF4−/− MEF were not capable of increasing VEGF mRNA levels following homocysteine treatment. In contrast, both the ATF4−/− and ATF+/− MEF were capable of inducing VEGF following glutamine starvation and anoxia. When ATF4 Wt protein was introduced by adenoviral infection, ATF4−/− MEF did increase VEGF mRNA expression in response to homocysteine treatment (Fig. 7*C*). In contrast, ATF4 DN had very little effect on the VEGF mRNA induction. This analysis also confirmed that induction of GRP78 mRNA expression was dependent upon ATF4 protein. Although ATF4 was not essential for GRP78 mRNA induction by homocysteine (Fig. 7*B*), ATF4 Wt protein did greatly increase the induction (Fig. 6*C*). This analysis also demonstrated that ATF4 protein, if not essential, is important to GADD153 mRNA induction in response to homocysteine. In conclusion, ATF4 plays a vital role in the induction of VEGF expression by MEF and ARPE-19 cultures in response to homocysteine treatment.

DISCUSSION

The present study includes the following novel findings. 1) Homocysteine induced the expression of VEGF mRNA in a time- and dose-dependent manner in a retinal-pigmented epithelial cell line, and D-and L-homocysteine had the same effect. 2) Homocysteine thiolactone and DTT also induced VEGF expression. 3) Homocysteine did not cause VEGF mRNA stabilization. 4) Homocysteine increased transcription from the *VEGF* gene. 5) Homocysteine, its thiolactone and DTT induced the expression not only of VEGF but also ER stress-responsive genes and the activation of IRE1 endonuclease activity (as measured by XBP-1 mRNA splicing). 6) Homocysteine treatment produced a transient phosphorylation of eIF2α followed by an increase in ATF4 protein level. 7) Inhibition of ATF4 function abrogated the VEGF response to homocysteine and to glutamine deprivation by ARPE-19 cells; and 8) ATF4−/− MEF were unable to induce VEGF in response to homocysteine treatment and the VEGF response was restored in the ATF4−/− MEF with expression of Wt ATF4.

Since VEGF was elevated by both the D- and L- isoforms of homocysteine, it is likely that the up-regulation of VEGF is a chemical effect that does not require metabolism of this amino acid. The up-regulation of VEGF by homocysteine thiolactone and DTT suggest that the thiol group may be key to the induction of VEGF. We suggest that the reductive potential of these compounds interferes with thiol oxidation and protein disulfide formation in the ER, thus inhibiting correct protein folding. Such is the case for DTT (46, 47). The amino acid L-cysteine had no significant effect on VEGF expression. In addition, homocysteine, thiolactone and DTT caused ER stress, whereas cysteine did not. A previous study also reported that cysteine did not cause ER stress (24). Whether this is the result of a lower reductive potential of the thiol group, cellular distribution, or cellular metabolism is not known.

Treatment of ARPE-19 cells with fresh medium containing homocysteine resulted in temporary increases in VEGF and GRP78 mRNA levels. The transient nature of these responses may be due to the fact that homocysteine is unstable in medium and thus causes a short-lived reductive insult. However, by measuring the amount of reactive free thiols in medium using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), we estimated that reduced homocysteine decays with a half-life of 25 h in the medium used (data not shown). Thus, an initial concentration of 10 mM homocysteine would still be ~6 mM by 16 h, at which time the VEGF response had effectively run its course.

Regardless of the cause, VEGF mRNA levels responded to homocysteine in a temporal manner that correlated closely to that of GRP78 mRNA. Because the response of GRP78 expression is known to be transcriptional, the correlation is consistent with the idea that transcription of VEGF is ER stress-responsive. This relation supports our past finding linking nutrient deprivation and chemical inducers of ER stress to the transcription of VEGF (11). mRNA decay rates indicated that homocysteine did not cause VEGF mRNA stabilization. In fact, mRNA decay curves showed a slight destabilization of VEGF mRNA by homocysteine. This is an unexpected result that should be interpreted with caution since measurement of VEGF decay below basal levels was difficult to achieve. We have interpreted the results only as indicative of a transcriptional up-regulation of VEGF, rather than a post-transcriptional mechanism. Nuclear run-on assays confirmed that homocysteine increased the rate of transcriptional initiation of the *VEGF* gene.

Furthermore, our results demonstrate that homocysteine causes a transient phosphorylation of eIF2α followed by an increase in ATF4 protein levels. This complements previous studies that indicated that ATF4 mRNA expression is induced by homocysteine (23, 48). ATF4 is a transcription factor that is expressed in response to nutrient and ER stress. ATF4 mRNA levels are increased in response to activation of the UPR and translation of the ATF4 protein is responsive to phosphorylation of eIF2α (26). Conditions that are known to activate eIF2α kinases include ER stress, amino acid deprivation, the presence of double-stranded RNA and heme deficiency. ATF4 may therefore be a common integrated response to signaling by stress-induced eIF2α kinases. The phosphorylation of eIF2α and the resultant increase in ATF4 precede temporally the up-regulation of VEGF mRNA, which is consistent with their involvement in *VEGF* gene regulation. A DN ATF4 adenoviral vector demonstrated that VEGF up-regulation in response to homocysteine and glutamine deprivation is dependent upon ATF4 function. Overexpression of the transcription factor ATF4 increased VEGF mRNA levels and expression of a DN mutant form of this transcription factor was capable of diminishing both homocysteine and glutamine starvation-induced VEGF mRNA levels in ARPE-19 cells. The present results also confirmed that AS and GRP78 expression are greatly affected by ATF4 activity. The role of ATF4 in control of GRP78 expression was just recently discovered (44).

The ATF4 DN mutant functions by competitively inhibiting the formation of functional complexes containing endogenous ATF4 proteins. Thus, the ability of the DN mutant to abrogate the VEGF response to homocysteine, nutrient stress, and anoxia supports the role of ATF4 in the up-regulation of VEGF. However, the sequestering of ATF4 binding partners in nonfunctional complexes with the DN mutant can also inhibit the normal function of those proteins. Thus, the negative effect of ATF4 DN overexpression must be interpreted with caution. Significantly, overexpression of wild-type ATF4 was sufficient to increase VEGF mRNA levels in unstressed cells, and did not inhibit the expression in nutrient and ER-stressed cells. This rules out a mechanism of action that relies on sequestration of other factors. Such a mechanism may, however, explain the negative effects of both wild-type and DN proteins on VEGF expression in anoxic cells. Anoxia has been shown to upregulate several members of the bZIP (basic/leucine zipper domain) transcription factor class, including ATF4 (49). Fig. 6 suggests that ATF4 and its binding partners play a role in the control of VEGF under a wide range of conditions.

The results of the ATF4 DN were confirmed in ATF4−/− MEF cultures. The absence of VEGF induction in the homocysteine treated ATF4−/− MEF suggest that ATF4 is an essential factor in homocysteine induced VEGF. The ability of Wt ATF4 protein expression to restore this response shows that ATF4 has a clear role in the up-regulation of VEGF under reductive stress. The

decrease in basal levels of VEGF in the ATF4−/− MEF suggests a broader role for ATF4 in basal VEGF expression. The ability of anoxia and glutamine deprivation to induce VEGF in the ATF4−/− MEF points toward complexity of VEGF regulation. Glutamine deprivation was expected to resemble homocysteine based on the effects of expressing the ATF4 DN in ARPE-19 cells. However, unlike homocysteine treatment, glutamine deprivation did not increase ATF4 protein levels in the ATF4+/− MEF. Thus, glutamine deprivation was seemingly able to induce VEGF expression by a mechanism independent of ATF4. Similar results were obtained with breast carcinoma cells.[2] These data indicate varying modes of transcriptional control of VEGF in different cell types and with different stresses.

Genes regulated by ATF4 include ones involved in amino acid import, metabolism and assimilation (27). We propose that ATF4 also up-regulates VEGF in order to satisfy increased nutritional needs of the cell under ER stress or to alleviate a nutritional deficiency that caused ER stress. In fact, the VEGF promoter region contains several sequences that match the AARE that are targets for binding of transcription factor complexes that contain ATF4. Our analysis of the DNA sequence of an 11.7-kb HindIII genomic fragment containing the human *VEGF* promoter (GenBank™ accession no. AL136131) has revealed several putative AARE elements (also referred to as nutritional stress response elements or NSRE). One of the NSRE sequences is identical to one found in the gene for the LIM-only protein DRAL promoter (TTTCCATCA) (50). Two other sites are both identical to the NSRE element within the *GADD153* promoter region (GTTTCACCA) (51). Another NSRE present in the second intron of the *VEGF* gene is identical to the element in the *AS* promoter (ATTACATCA) (45).

In summary, the results of the present study indicate that expression of the pro-angiogenic factor VEGF is increased by homocysteine and other thiol-containing reductive compounds. Our data provide evidence that the effect of homocysteine on VEGF expression is due to ATF4-dependent activation of VEGF transcription. ATF4 plays a role in the control of VEGF transcription in cells exposed to abnormally high homocysteine concentrations. These results suggest a mechanism by which hyperhomocysteinemia could contribute to the development of retinal neovascularization through stimulation of VEGF expression by retinal cells. However, we are not aware of any direct evidence that homocysteine levels are increased in the vitreous or in retinal cells during development of any retinal disease. The only indirect suggestion that this may occur was provided by Naggar *et al.* (34) who demonstrated that expression of the reduced-folate transporter RFT-1 was down-regulated in RPE cells by high glucose and was reduced in the RPE of diabetic mice. Thus suggesting a mechanism whereby hyperglycemia could lead to elevated intracellular homocysteine levels in the RPE. The involvement of ATF4 gives direct evidence linking ER stress and angiogenesis. This finding could have broader applications in many other angiogenesis-driven pathologies. Further studies are aimed at determining essential ATF4 binding sites within the *VEGF* promoter as well as the ATF4 binding partner that is involved in this induction.

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REFERENCES

1. Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. (1983) *Science* **219,** 983–985
2. Gerber, H. P., Hillan, K. J., Ryan, A. M., Kowalski, J., Keller, G. A., Rangell, L., Wright, B. D., Radtke, F., Aguet, M., and Ferrara, N. (1999) *Development* **126,** 1149–1159
3. Pulido, J. S. (1999) *The Present Problem and the Future*, Vol. 12, p. 564, Ophthalmology Clinics of North America, W. B. Saunders Company, Philadelphia
4. Aiello, L. P., and Wong, J. S. (2000) *Kidney Int. Suppl.* **77,** S113–S119
5. Aiello, L. P., Northrup, J. M., Keyt, B. A., Takagi, H., and Iwamoto, M. A. (1995) *Arch. Ophthalmol.* **113,** 1538–1544
6. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992) *Nature* **359,** 843–845
7. Lu, M., Kuroki, M., Amano, S., Tolentino, M., Keough, K., Kim, I., Bucala, R., and Adamis, A. P. (1998) *J. Clin. Investig.* **101,** 1219–1224
8. Tilton, R. G., Kawamura, T., Chang, K. C., Ido, Y., Bjercke, R. J., Stephan, C. C., Brock, T. A., and Williamson, J. R. (1997) *J. Clin. Investig.* **99,** 2192–2202
9. Sone, H., Kawakami, Y., Okuda, Y., Kondo, S., Hanatani, M., Suzuki, H., and Yamashita, K. (1996) *Biochem. Biophys. Res. Commun.* **221,** 193–198
10. Satake, S., Kuzuya, M., Miura, H., Asai, T., Ramos, M. A., Muraguchi, M., Ohmoto, Y., and Iguchi, A. (1998) *Biol. Cell* **90,** 161–168
11. Abcouwer, S. F., Marjon, P. L., Loper, R. K., and Vander Jagt, D. L. (2002) *Invest. Ophthalmol. Vis. Sci.* **43,** 2791–2798
12. Agardh, E., Hultberg, B., and Agardh, C. D. (2000) *Scand. J. Clin. Lab. Invest.* **60,** 169–174
13. Agardh, C. D., Agardh, E., Andersson, A., and Hultberg, B. (1994) *Scand. J. Clin. Lab. Investig.* **54,** 637–641
14. Neugebauer, S., Baba, T., Kurokawa, K., and Watanabe, T. (1997) *Lancet* **349,** 473–474
15. Looker, H. C., Fagot-Campagna, A., Gunter, E. W., Pfeiffer, C. M., Venkat Narayan, K. M., Knowler, W. C., and Hanson, R. L. (2003) *Diabetologia* **46,** 766–772
16. de Bruijne, E. L., Keulen-de Vos, G. H., and Ouwendijk, R. J. (1999) *Ann. Intern. Med.* **130,** 78
17. Cahill, M. T., Stinnett, S. S., and Fekrat, S. (2003) *Am. J. Ophthalmol.* **136,** 1136–1150
18. Axer-Siegel, R., Bourla, D., Ehrlich, R., Dotan, G., Benjamini, Y., Gavendo, S., Weinberger, D., and Sela, B. A. (2004) *Am. J. Ophthalmol.* **137,** 84–89
19. Moore, P., El-sherbeny, A., Roon, P., Schoenlein, P. V., Ganapathy, V., and Smith, S. B. (2001) *Exp. Eye Res.* **73,** 45–57
20. Lentz, S. R., and Sadler, J. E. (1991) *J. Clin. Investig.* **88,** 1906–1914
21. Lentz, S. R., and Sadler, J. E. (1993) *Blood* **81,** 683–689
22. Kokame, K., Kato, H., and Miyata, T. (1996) *J. Biol. Chem.* **271,** 29659–29665
23. Outinen, P. A., Sood, S. K., Pfeifer, S. I., Pamidi, S., Podor, T. J., Li, J., Weitz, J. I., and Austin, R. C. (1999) *Blood* **94,** 959–967
24. Werstuck, G. H., Lentz, S. R., Dayal, S., Hossain, G. S., Sood, S. K., Shi, Y. Y., Zhou, J., Maeda, N., Krisans, S. K., Malinow, M. R., and Austin, R. C. (2001) *J. Clin. Investig.* **107,** 1263–1273
25. Talloczy, Z., Jiang, W., Virgin, H. W. t., Leib, D. A., Scheuner, D., Kaufman, R. J., Eskelinen, E. L., and Levine, B. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 190–195
26. Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) *Mol. Cell* **6,** 1099–1108
27. Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D. F., Bell, J. C., Hettmann, T., Leiden, J. M., and Ron, D. (2003) *Mol. Cell* **11,** 619–633
28. Masuoka, H. C., and Townes, T. M. (2002) *Blood* **99,** 736–745
29. Chambers, J. C., Ueland, P. M., Wright, M., Dore, C. J., Refsum, H., and Kooner, J. S. (2001) *Circ. Res.* **89,** 187–192
30. Abcouwer, S. F., Schwarz, C., and Meguid, R. A. (1999) *J. Biol. Chem.* **274,** 28645–28651
31. Chomcynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
32. Kevil, C. G., Walsh, L., Laroux, F. S., Kalogeris, T., Grisham, M. B., and Alexander, J. S. (1997) *Biochem. Biophys. Res. Commun.* **238,** 277–279
33. Hargrove, J. L., Hulsey, M. G., and Beale, E. G. (1991) *Bioessays* **13,** 667–674
34. Naggar, H., Fei, Y. J., Ganapathy, V., and Smith, S. B. (2003) *Exp. Eye Res.* **77,** 687–697
35. Kane, K. K., Hawkins, D. E., Pulsipher, G. D., Denniston, D. J., Krehbiel, C. R., Thomas, M. G., Petersen, M. K., Hallford, D. M., Remmenga, M. D., Roberts, A. J., and Keisler, D. H. (2004) *J. Animal Sci.* **82,** 283–291
36. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2509–2514
37. He, C. H., Gong, P., Hu, B., Stewart, D., Choi, M. E., Choi, A. M., and Alam, J. (2001) *J. Biol. Chem.* **276,** 20858–20865
38. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) *Cell* **107,** 881–891
39. Harding, H. P., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000) *Mol. Cell* **5,** 897–904
40. Levy, A. P., Levy, N. S., Iliopoulos, O., Jiang, C., Kaplin, W. G., Jr., and Goldberg, M. A. (1997) *Kidney Int.* **51,** 575–578
41. von Marschall, Z., Cramer, T., Hocker, M., Finkenzeller, G., Wiedenmann, B., and Rosewicz, S. (2001) *Gut* **48,** 87–96
42. Dibbens, J. A., Miller, D. L., Damert, A., Risau, W., Vadas, M. A., and Goodall, G. J. (1999) *Mol. Biol. Cell* **10,** 907–919
43. Claffey, K. P., Shih, S. C., Mullen, A., Dziennis, S., Cusick, J. L., Abrams, K. R., Lee, S. W., and Detmar, M. (1998) *Mol. Biol. Cell* **9,** 469–481
44. Luo, S., Baumeister, P., Yang, S, Abcouwer, S. F., and Lee, A. S. (2003) *J. Biol. Chem.* **278,** 37375–37385
45. Siu, F., Bain, P. J., LeBlanc-Chaffin, R., Chen, H., and Kilberg, M. S. (2002) *J. Biol. Chem.* **277,** 24120–24127
46. Losch, A., and Koch-Brandt, C. (1995) *J. Biol. Chem.* **270,** 11543–11548
47. Negroiu, G., Dwek, R. A., and Petrescu, S. M. (2000) *J. Biol. Chem.* **275,** 32200–32207
48. Kokame, K., Kato, H., and Miyata, T. (1998) *Methods (Duluth)* **16,** 434–443
49. Estes, S. D., Stoler, D. L., and Anderson, G. R. (1995) *Exp. Cell Res.* **220,** 47–54
50. Okada, T., Yoshida, H., Akazawa, R., Negishi, M., and Mori, K. (2002) *Biochem. J.* **366,** 585–594
51. Ma, Y., Brewer, J. W., Diehl, J. A., and Hendershot, L. M. (2002) *J. Mol. Biol.* **318,** 1351–1365

[2] Y. Bobrovnikova-Marjon and S. F. Abcouwer, unpublished data.